I hereby certify that this correspondence is being deposited with the U.S. Postal Service as Express Mail, Airbill No. EV336629649US, in an envelope addressed to: MS Appeal Brief - Patents, Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450, on the date shown below.

Dated: January 30, 2004 Signature: _____ (E. Thomas Wheelock)

Docket No.: 356972020100
(PATENT)

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Patent Application of:

Hirokazu SUGIHARA et al.

Application No.: 08/913,811 Confirmation No.: 7552

Filed: September 24, 1997 Art Unit: 1645

For: METHOD FOR MEASURING PHYSICOCHEMICAL PROPERTIES OF TISSUES OR CELLS, METHOD FOR EXAMINING CHEMICALS, AND APPARATUS THEREFOR

Examiner: P. Baskar

APPELLANTS' BRIEF

MS Appeal Brief - Patents
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

This is an appeal from the final rejection of claims 12, 14, and 16, in Paper No. 35, mailed December 31, 2002. The Notice of Appeal was filed on June 30, 2003.

The fees required under § 1.17(f) and any required petition for extension of time for filing this Brief and fees therefore, are dealt with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This Brief is transmitted in triplicate.

This Brief contains items under the headings required by 37 C.F.R. § 1.192 and M.P.E.P. § 1206.

I. REAL PARTY IN INTEREST

The real party in interest for this appeal is: Matsushita Electric Industrial Ltd. of Osaka, Japan.

II. RELATED APPEALS AND INTERFERENCES

There are no other appeals or interferences which will directly affect or be directly affected by or have a bearing on the Board's decision in this appeal.

III. STATUS OF CLAIMS

A. Total Number of Claims in Application

There are three claims pending in application.

B. Current Status of Claims

1. Claims canceled: 13 and 15

2. Claims withdrawn from consideration but not canceled: 1-11

3. Claims pending: 12, 14, and 16

4. Claims allowed: 0

5. Claims rejected: 12, 14, and 16

C. Claims On Appeal

The claims on appeal are claims 12, 14, and 16 and are included in the **Attachment A**.

IV. STATUS OF AMENDMENTS

The claims on appeal were last amended prior to and were the object of the Final Rejection (Paper No. 36 – apparently partially mis-numbered in the record as Paper No. 35, but in

any case mailed by the PTO on December 31, 2002). No amendments have been made to the claims since that Final Rejection.

Appellants filed an Response to the Final Rejection on February 28, 2003. The Examiner responded to the Amendment After Final Rejection in an Advisory Action, Paper No. 40, mailed November 19, 2003. In the Advisory Action, the Examiner indicated that Appellants' arguments were considered but the rejection stood.

V. SUMMARY OF INVENTION

The claimed portion of the technology disclosed in the specification is a procedure for measuring electrical properties of a muscle tissue sample or a neural tissue sample in response to an added chemical material. It is a laboratory-type process practiced upon samples taken from an animal. A "neural sample" would typically be a sample taken from a brain although it need not be. The term "muscle sample" generally needs no additional explanation. The method requires the placement of a sample upon a multi-electrode surface that is able both to stimulate the tissue sample and to measure the electrical activity of that sample. It is an inherent property of such tissues that when still "living" in a laboratory sense, the tissues pass electricity in certain patterns through conduction passageways in the tissues. These currents, observed as electric potentials or voltage by the sensors, are measurable quantities that are indicia of the tissue itself. This phenomena in muscles was probably first observed by a crowd of Italian scientists the late 1700's and early 1800's. The anatomist Luigi Galvani noticed that the muscles in frog legs, not otherwise connected to the relevant frog, contracted when contacted with a current source. Galvani subsequently found that electric stimulation of a frog's heart muscle caused muscular contraction. Allessandro Volta, in attempting to disprove the conclusions of Galvani, invents the voltaic pile -- the first of the practical batteries. Carlo Matteucci, professor of physics at the University of Pisa, then demonstrated the presence of a small current with each heart beat using a cut nerve in a frog's leg as an electrical sensor and the twitching of the associated frog muscle as the visual sign of electrical activity. In any case, today such electrical activity is routinely monitored in the human heart by the device producing electrocardiograms and in the human brain by devices producing electroencephalograms (EEG).

Returning to the Appellants' claimed technology, the process then involves a "before" measurement of that sample tissues' electrical activity, the eventual introduction of a chemical substance to the tissue sample in some fashion, and the subsequent or "after" measurement of electrical properties of the tissue sample itself. The comparison of the "before" and "after" measurements allow the user to determine the effect of the chemical material upon that tissue sample. Measurement of the electrical activity is both desired and possible at a time when the observed effect would be considered a "chronic" effect as that term is used in medicine today.

This methodology allows the process user to perform such analysis as determining whether a particular chemical substance has long term effects on the brain, e.g. perhaps causing diseases or addiction, by simply observing the changes in electrical activity of certain portions of a neural tissue sample over an extended period of time.

The method involves the use of a detector having a number of electrodes on a substrate. One will note that the detector is put together in such a way that it is able to contact the tissue sample with a number of electrodes and is able both to apply an electric stimulus to the tissue sample and to measure the electrical properties of the tissue sample. A typical device is shown in Figures 1-3 of the application. This type of a procedure is shown, for instance, in Example 1. Example 1 shows the placement of "a slice of the cerebral cortex of a rat...used as nervous tissue" on (or into) the multi-electrode device and further shows the effect of introducing methamphetamine (as a "chemical substance") to the sample. The type of culturing to preserve the viability of the tissue sample is discussed at length in Example 1 but suffice it to say that section 4 of Example 1 (beginning at page 33, bottom of the page), indicates there is a significant difference between the culturing used on "Nervous Tissue or Nervous Cells". It is these tissue "samples" that are required by the procedure of these claims. It is the measurement of the electrical activities of the tissue - - not of some resulting effect upon the fluid in the neighborhood of the surface of the multi-electrode array -- that is watched in this claimed method.

VI. ISSUES

The issues in this appeal are as follows:

1. Whether claims 12, 14, and 16 are properly rejected under 35 U.S.C. 103(a) as unpatentable over Gahwiler et al. (Neuroscience, 1982, 7; 1243-1256) in view of Gross et al. (J. of Neuroscience Methods 5:13-22, 1982), and

2. Whether claims 12, 14, and 16 are properly rejected under 35 U.S.C. 103(a) as unpatentable over Gahwiler et al. (noted above) in view of Giaever et al. (U. S. Patent No. 5,187,096).

(Copies of the three references are found in **Attachment B**.)

VII. GROUPING OF CLAIMS

For purposes of this Appeal Brief, claims 12, 14, and 16 rise and fall together.

VIII. ARGUMENTS

This is a fairly straightforward appeal having but a few straightforward points. The first point: none of the cited references show the placement of a tissue sample on a multi-electrode array. It is to be recalled that a "tissue sample" is something different from either individual cells or clumps of later recultured cells in that the latter lack the anatomical structure found in a "tissue sample." Secondly, there are no clear bases articulated in the Final Rejection for combining the primary and secondary references and certainly the references are silent as to such reasons. Finally, since the various devices in the references do not measure electrical properties of the tissue sample at multiple sites, none of the references consequently measure those multiple effects on the tissue sample's electrical activity, whether those effects be either short term or chronic.

Gahwiler et al. in view of Gross et al.

Claims 12, 14, and 16 stand rejected under 35 U.S.C. 103(a) as unpatentable over Gahwiler et al. (Neuroscience, 1982, 7; 1243-1256) in view of Gross et al. (J. of Neuroscience Methods 5:13-22, 1982). The rejection over which this appeal is made is considered to be that recited in the Office Action dated December 31, 2002 (Paper No. 35 [or 36]). Specifically the final rejection is believed to be:

> "Gahwiler et al. 1982, teach a method of testing the effect of chemical substances (acetylcholine) on neuronal tissue (hippocampal sections) and measuring the electrical properties (see experimental procedures on page 1243 and 1244) before and after addition of said substance (see results and figures). Although the prior art used standard electrophysiological techniques for recording the electrical properties, the prior art specifically does not teach providing a detector comprising plurality of microelectrodes on a substrate for contacting the tissue sample (i.e., the device or apparatus).
>
> "Gross et al teach an apparatus (see material and methods/figures) for observing a physical and chemical property of a tissue or cells comprising providing photo etched electrodes integrated into the floor of a tissue culture chamber (i.e. providing a substrate with planar electrodes disposed on the same plane as the substrate) and a cell culturing means. (page 13). Gross et al teach recording the electrophysiological potentials with electrodes integrated into the tissue culture plate would allow the long term monitoring of neuronal activity. It would have been prima facie obvious to one of ordinary skill in the art at the time that the invention was made to us e the apparatus designed by Gross in a method of Gahwiler et al to measure the electrical properties before and after addition of chemical substances because Gross et al suggests that the apparatus disclosed in obviously designed for long term cultures. The motivation to use this apparatus to achieve the obviously designed for long term cultures. The motivation to use this apparatus to achieve the obvious benefits is clearly suggested by Gross (see page 21, last paragraph). Therefore, it would have been *prima facie* obvious to one having ordinary skill in the art at the time the invention was made to use the apparatus as taught by Gross

> et al for measuring and comparing waveforms or electrical properties of neural tissue before and after the addition of chemical substances as taught Gahwiler et al because the apparatus is designed to measure the effect of different concentrations of the chemical substance on tissue and comparing the electrical properties of long term cultures."

In response to arguments made by Appellants, the Examiner expanded upon the rejection in the Advisory Action thusly:

> "Applicant states that Gahwiler do not measure a chronic effect (i.e., test compound) on tissue sample but teaches measurement of cell potentials from rat hippocampus that has been cultured using the roller-tube technic and Gross does not teach neural tissue sample but teaches disassociated neurons on a multi-electrode plate.
>
> "It is the examiner's position that the term "chronic" is a relative term without any lower boundaries and not defined by either the claim or by the specification. Gahwiler use neural tissue and Gross suggests the apparatus disclosed is obviously designed for long-term cultures. Therefore, the teachings of prior art established a prima facie obviousness. The examiner has clearly established a prima facie obviousness using the basic criteria, suggestion and reasonable expectation of success. Gahwiler et al 1982, teach a method of testing the effect of chemical substances (acetylcholine) on neuronal tissue (hippocampal sections) and measuring the electrical properties (see experimental procedures on page 1243 and 1244) before and after addition of said substances (see results and figures). The term "chronic" is not defined by the claim; the specification does not provide a standard for ascertaining the requisite degree. Therefore, the prior art teaches a method of testing the chronic effect of compounds in a neural tissue culture. Further, Gross et al teach an apparatus (see material and methods/figures) and a method for observing a physical and chemical property of a tissue or cells comprising providing photo etched electrodes integrated into the floor of a tissue culture chamber (i.e. providing a substrate with planar electrodes disposed on the same plane as the substrate) and a method of cell culturing means. (page 13).
>
> "Gross et al teach recording the electrophysiological potentials with electrodes integrated into the tissue culture plate would allow the long term monitoring of neuronal activity. The examiner is aware that Gross did not use tissue but the claims were rejected under 35 U.S.C. 103 and not under 35 U.S.C. 102. Therefore, it would have been prima facie obvious to one of ordinary skill in the art at the time that the invention was made to use the apparatus in a method designed by Gross to teachings of Gahwiler et al

> to measure the electrical properties before and after addiction of chemical substances to neural tissue sample because Gross et al suggests that the apparatus disclosed is obviously designed for long term cultures. The motivation to use this apparatus and a method to achieve the obvious benefits is clearly suggested by Gross (see page 21, last paragraph). Thus the teaching of the prior art make the claimed invention obvious. Therefore, the rejection is maintained."

Appellants have consistently disagreed with this rejection and typically for the reasons discussed above.

The Gahwiler reference shows at page 1243 and the EXPERIMENTAL PROCEDURES that hippocampal cultures were prepared by cutting slices of rat brain, embedding those slices in a plasma clot, and then culturing them for 3-11 weeks in a device using a "roller tube technique." The article goes on to note that the resulting roller-tube cultures "flattened during the first four weeks to such an extent that individual nerve cells can easily be viewed with phase contrast microscopy." Consequently, the roller tube cultures arguably provide a "tissue sample" albeit many weeks later. However, there is no indication that a multi-electrode measuring device was used and indeed, the EXPERIMENTAL PROCEDURES section indicates that only "standard electrophysiological techniques were used." This statement means that, at that time, individual electrodes were the norm. Specifically, the penultimate paragraph of the EXPERIMENTAL PROCEDURES section mentions that various other electrodes (stimulation, ACh introduction, etc.) "could be positioned independently of the recording electrode." To repeat, the article says "the" recording electrode.

The requirements in claim 12 of providing a detector having a "plurality of electrodes" and the further requirement of contacting the "neural or muscle tissue sample" with the "plurality of electrodes" are not there by accident. Multiple electrodes allow measurement of electrical activity in the tissue sample at the same time at multiple sites. That the relationship amongst these various measurements may be important to determination of the type of chemical substances assessed is a feature not mentioned in the Gahwiler et al. disclosure. Of course, since the tests

discussed in the article were conducted using "the" recording electrode, it could not have been so mentioned.

In a similar vein, there is no particular mention of the concept of maintaining the neural or muscle tissue sample in conjunction with the single recording electrode in such a way that one is able to measure electrical properties of the neural or muscle tissue sample at a time which measures "chronic response." Specifically, the longest measurement found in the Gahwiler et al. article appears to the 90 seconds time found in Figure 8. A period of 90 seconds can, in no way, be considered "chronic." A time of 90 seconds is, by any reasonable medical standard, a time at which an "acute" measurement is taken. Gahwiler et al. clearly uses a older, earlier generation type of measuring equipment. To do any measurement that could be considered to show a "chronic" result, Gahwiler would find it necessary to return the various tissue slices to the roller tube device for further culturing. At a still later time, the slices would re-make the journey to the measurement site to again be probed by "the" recording electrode. The difficulty in repositioning "the" recording probe in the same place on the sample after the additional roller-tube culturing, is staggering. Again, the slices under study there were from rats having an age of from seven to ten days. The samples are not large. But, to compare those measured voltages on a "chronic" basis using the Gahwiler et al method, that is the task.

It is clear that the work described in the Gahwiler et al article did not include such an effort. It is also not apparent that such a task would be possible understanding the difficulty of placing and then later re-placing such a probe in multiple sites in a slice taken from a seven to ten day old rat.

In sum, Gahwiler et al does not show the placement of a tissue slice on a multi-electrode detector, it does not suggest any value in measuring electrical activities at different times that are much more than a minute or two apart, and it does not show any reason to consistently measure the electrical activities of the tissue slice at a same spot.

The Gross et al. reference describes a device that cultures mice spinal tissue cells on a multi electrode plate. The procedure used with the Gross et al. device does not measure the

electrical activity of a neural or muscle tissue sample. The activity measured is only that of individual or re-aggregated mouse spinal cells. No suggestion of the placement of tissue slices into the Gross et al. device is to be found in the article. Indeed the article (on the first page), the goal of the study was to achieve "recordings from mammalian CNS neurons cultured on the electrode surface." (emphasis added) That is not a teaching to use slices from mammalian sources.

The Examiner suggests that a goal of Gross et al is to achieve procedures for long term monitoring. That is a goal found at the end of the first paragraph on page 14. however, the goal relates to "mouse spinal neurons," not to whole tissue samples. In assessing the achievements of the project, the authors note at page 21 that "the multi electrode plates used in these experiments are obviously designed for the long term, simultaneous recording of spike activity from cultured neuronal monolators in which the active units in most of their inner connections can be identified and observed." Again, this comment does suggest the use of this device on a tissue slice. The authors go on to say that although "preliminary data show healthy-looking cultures can be maintained ... for over three weeks, we cannot yet make any statements about signal acquisition during that period of time. Monolayers and presumably the surfaces of tissue micro fragment are not morphologically stable."

The journal article complains that movement of the cultured material is a significant problem. This likely means that the data may not be particularly useful. If the Gross et al authors weren't recommending their device and the allied procedure, why would one having ordinary skill in this art have taken the procedure found in the Gross et al reference and do something with it not apparently envisioned -- a process such as that shown in Gahwiler et al.? What confidence would that one of ordinary skill have that the combined processes would produce useful results? The references are ill-combined in the Final Rejection and the rejection should be withdrawn.

In sum, the procedures shown in Gahwiler et al. and Gross et al., even as combined in the Office Action, would not produce a method such as required by the independent claim 12. Indeed there are significant reasons why one would not practice the procedure Gahwiler et al. in the device of Gross et al. since the Gross et al. considers the device to be an unlikely candidate for long term measurement of even those samples that are derived from neural or muscle tissue.

The rejection should be reversed.

2.) Gahwiler et al. in view of Gross et al.

Claims 12, 14, and 16 stand rejected under 35 U.S.C. 103(a) as unpatentable over Gahwiler et al. (Neuroscience, 1982, 7; 1243-1256) in view of Giaever et al. (U.S. Patent No. 5,187,096). In support of the final rejection, the Examiner stated:

> "Giaever et al teach an apparatus (see claims) for observing a physical and chemical property of a tissue or cells comprising plurality of electrodes integrated into the floor of a tissue culture chamber (i.e. providing a substrate with planar electrodes disposed on the same plane as the substrate) and cell culturing (column 2, Summary of the invention) means. Giaever et al teach by using this apparatus, the activities of cultured cells that are attached to the surfaces could be followed continuously in real time. The recording of extracellular electrophysiological potentials with electrodes integrated into the tissue culture plate would allow the long term monitoring of cell activity to change in the physical environment and drugs (column 3 and 4). It would have been prima facie obvious to one of ordinary skill in the art at the time that the invention was made to use the apparatus designed by Giaever et al in a method of Gahwiler et al to measurer the electrical properties before and after the addition of chemical substances because Giaever et al suggests that this apparatus is obviously designed for long term cultures. The motivation to use this apparatus to achieve the obvious benefits is clearly suggested y Giaever et al. (see column 3, lines 23-55). therefore it would have been *prima facie* obvious to one having ordinary skill in the art at the time the invention was made to use the apparatus as taught by Giaever et al for measuring and comparing waveforms or electrical properties of neural tissue before and after the addition of chemical substances as taught by Gahwiler et al because the apparatus designed by Giaever is fore measuring the effect of different concentration of the chemical substances on tissue and comparing the electrical properties of long term cultures."

In response to Appellants' arguments made after the Final Rejection, the Examiner added the following in the Advisory Action:

> "Applicant states that Gahwiler do not measure a chronic effect (i.e. of test compound) on tissue sample but teaches a measurement of cell potentials from rat hippocampus that has been cultured using the roller-tube technic and Giaever et al (U.S. Patent No. 5,187,096) do not teach a device that measures an electrical property of tissue.
>
> "The Gahwiler has been discussed supra. The examiner is aware that Giaever did not use tissue but the claims are rejected under 35 U.S.C. 103 and do under 35 U.S.C. 102. Therefore, the teachings of Gahwiler using a neural tissue for measuring the effect of compound in a method using Giaever apparatus makes the claimed invention obvious over the prior art as explained. Therefore, the rejection is maintained".

The rejection of the claims under 35 USC as rendered obvious by Gahwiler et al. in view of Giaever et al. is inappropriate. The Gahwiler et al. article has been discussed above. The Giaever patent makes no mention of a process that measures either electrical activity or an electrical property of a tissue sample. Giaever mentions only the measurement of impedance, in this instance a specific property of the fluid surrounding the individual cells found in the Giaever electrode array. There is no indication in Giaever that there is any reason to measure completely a different property -- electrical properties of the neural or muscle tissue sample.

Furthermore, claim 12 requires that the neural or muscle tissue sample be contacted "with the plurality of electrodes." It is not apparent from reading of the Giaever et al. reference that in any instance is there a particular sample may be in contact with two electrodes at the same time. Each electrode in the Giaever et al. reference is each isolated -- one to a well. See, for instance, Figure 1 and note that the well shows a "single cell culture cell assembly" having a "cylinder 12...[with] a large upper electrode 18 containing a cylindrical hole 20 which also...includes a small hole 28 positioned within the hole 20 for each well assembly 10, to expose a small electrode area 30 of strip 24." See column 5, lines 35-50. It is unclear how one of ordinary skill in this art would place a single neural or muscle tissue sample in the multiple electrode sites found in this disclosure

and expect it to operate in variously the manner described in the Giaever patent, the Gahwiler article, or the claimed method.

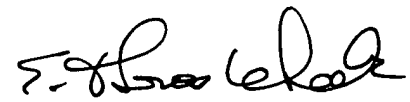
The final rejection should be reversed.

IX. CLAIMS INVOLVED IN THE APPEAL

A copy of the claims involved in the present appeal is attached as Appendix A.

Dated: January 30, 2004

Respectfully submitted,

By 

E. Thomas Wheelock
Registration No.: 28,825
MORRISON & FOERSTER LLP
755 Page Mill Road
Palo Alto, California 94304

(650) 813-5739

CLAIMS OF SER. NO. 08/913,811 ON APPEAL

12. A method of testing the chronic effect on neural or muscle tissue samples of chemical substances comprising:

providing a detector, wherein the detector comprises a plurality of microelectrodes on a substrate configured to contact the tissue sample and apply an electric stimulus to the tissue sample;

contacting said neural or muscle tissue sample with the plurality of electrodes;

measuring the electrical properties of the neural or muscle tissue sample;

adding said chemical substance to the neural or muscle tissue sample;

measuring the electrical properties of the neural or muscle tissue sample at a time which measures chronic response to said chemical substance; and

comparing said electrical properties before and after said addition of said chemical substance to determine whether said added chemical substance has had an influence on the neural or muscle tissue sample.

14. The method of claim 12 for testing the effect on neural or muscle tissue samples of chemical substances as medicines, wherein the step of adding chemical substance to the neural or muscle tissue sample comprises adding said chemical substance in a selected concentration to the neural or muscle tissue sample.

16. The method of claim 12 for testing the effect on neural or muscle tissue samples of chemical substances as medicines, wherein the chronic measuring step takes place at least three days after said addition step.

XP 2037335

Elsevier Biomedical Press

Recording of spontaneous activity with photoetched microelectrode surfaces from mouse spinal neurons in culture

Guenter W. Gross, Ann N. Williams and Jen H. Lucas

Department of Biology, The Texas Woman's University, Denton, TX 76204 (U.S.A.)

(Received April 7th, 1981)
(Revised version received July 20th, 1981)
(Accepted July 27th, 1981)

Key words: photoetched electrodes — cell culture — laser deinsulation — mouse spinal neurons — multielectrode surface

A matrix of photoetched gold conductors integrated into the floor of a tissue culture chamber has been used to record from mammalian spinal cord neurons grown on the insulation layer of the multielectrode plate. Spontaneous activity has been monitored from tissue microfragments less than 150 μm in diameter and from thin sheets of spinal cell aggregates. Maximum spike amplitudes of 360 μV with signal-to-noise ratios of 8 : 1 have so far been achieved and the spontaneous activity maintained for several days. Recording electrode impedances measured between 4 and 7 MΩ at 1 kHz. Conductor tips were deinsulated with laser pulses that formed shallow craters 2 μm deep and 12 μm in diameter. Addition of colloidal gold or platinum black was not necessary to achieve satisfactory recordings.

Introduction

The recording of extracellular electrophysiological potentials with photoetched electrodes integrated into the floor of the tissue culture chamber is an attractive technique that should allow the long-term monitoring of neuronal activity in explant as well as dispersed cell culture. The main advantages of this new technique are the potential for simultaneous recording from and stimulation of a large number of neurons, the elimination of vibrations between recording electrode and signal source, the ability to carry out experiments in closed, sterile chambers, and the ability to carry out high-power microscopy during recording.

Chambers containing photoetched electrodes have been used to record field potentials from cultured cardiac muscles (Thomas et al., 1972), to monitor simultaneous single unit activity from snail ganglia (Gross, 1979; Gross et al., 1977) and to record evoked action potentials from dissociated mammalian ganglionic neurons (Pine, 1980). However, no laboratory has so far achieved recordings from mammalian CNS neurons cultured on the electrode surface. This important step in

demonstrating the usefulness of this new electrophysiological technique has been hindered by a variety of neurobiological and technical difficulties such as insulation breakdown after days under warm saline, electrode reinsulation by vigorous glia growth, poor cellular adhesion to the most stable insulation materials and the monitoring of signals from the relatively small CNS neurons. It is the purpose of this paper to report recently developed procedures that have allowed the monitoring of mouse spinal neurons for several days with photoetched electrodes.

Materials and methods

Insulation of multimicroelectrode surface

From our experience, hydrophilic insulation materials swell in saline and usually reveal an unacceptable drop in shunt impedance within minutes to hours after exposure to the culture medium. The insulation material we have found most stable under saline at 37°C is a highly hydrophobic polysiloxane resin (Dow Corning DC 648). Deinsulation of the tips of the gold electrodes in the area of the recording matrix was performed using single shots from an ultraviolet laser (337.1 nm) at an energy density of 4 $\mu J/\mu m^2$ (Gross, 1979). Usually 2–4 electrodes were left insulated so that shunt impedances might be checked periodically as an indicator of insulation stability (Gross, 1979). Electrode impedances (Z) ranged from 4 to 12 MΩ with an average Z of 6 MΩ. Recording electrode impedances have so far not exceeded 7 MΩ.

Preparation of insulated surface for culturing

Due to its hydrophobic nature the polysiloxane resin does not permit good cell adhesion. However, a 1 s exposure of the recording area to temperatures above 1500°C produces a hydrophilic oxidation layer to which cells adhere more readily.

Tissue culture

Spinal cord tissue was isolated and dissociated from 13- to 15-day mouse embryos (GIBCO HA/ICR) according to the procedure of Ransom et al. (1977) with the following modifications. Seven to twelve spinal cords were pooled in 1 ml Hanks BBS containing 2% penicillin–streptomycin during dissection. The tissue was transferred to a dry, sterile petri dish and minced with scalpels. It was then transferred to 1.5 ml of Eagle's Minimum Essential Medium (GIBCO, Earle's liquid), fortified with 10% heat-inactivated horse serum (GIBCO) and 10% fetal calf serum (GIBCO), and buffered for 5% CO_2 atmosphere. The tissue was further dissociated by trituration with Pasteur pipettes (10–12 times). Of the 3 ml final suspension volume 1 ml was plated directly on the multimicroelectrode plate (MEP) and was confined by a 1 mm thick silicone gasket forming a plating area of 600 mm^2. Fluorodeoxyuridine (FdU) plus uridine was added 48 h after plating in the concentrations recommended by Ransom et al. (1977). The first medium changes were carried out within 3–6 days after seeding with MEM containing 10% horse serum. In all medium changes only 50% of the old medium was removed and replaced.

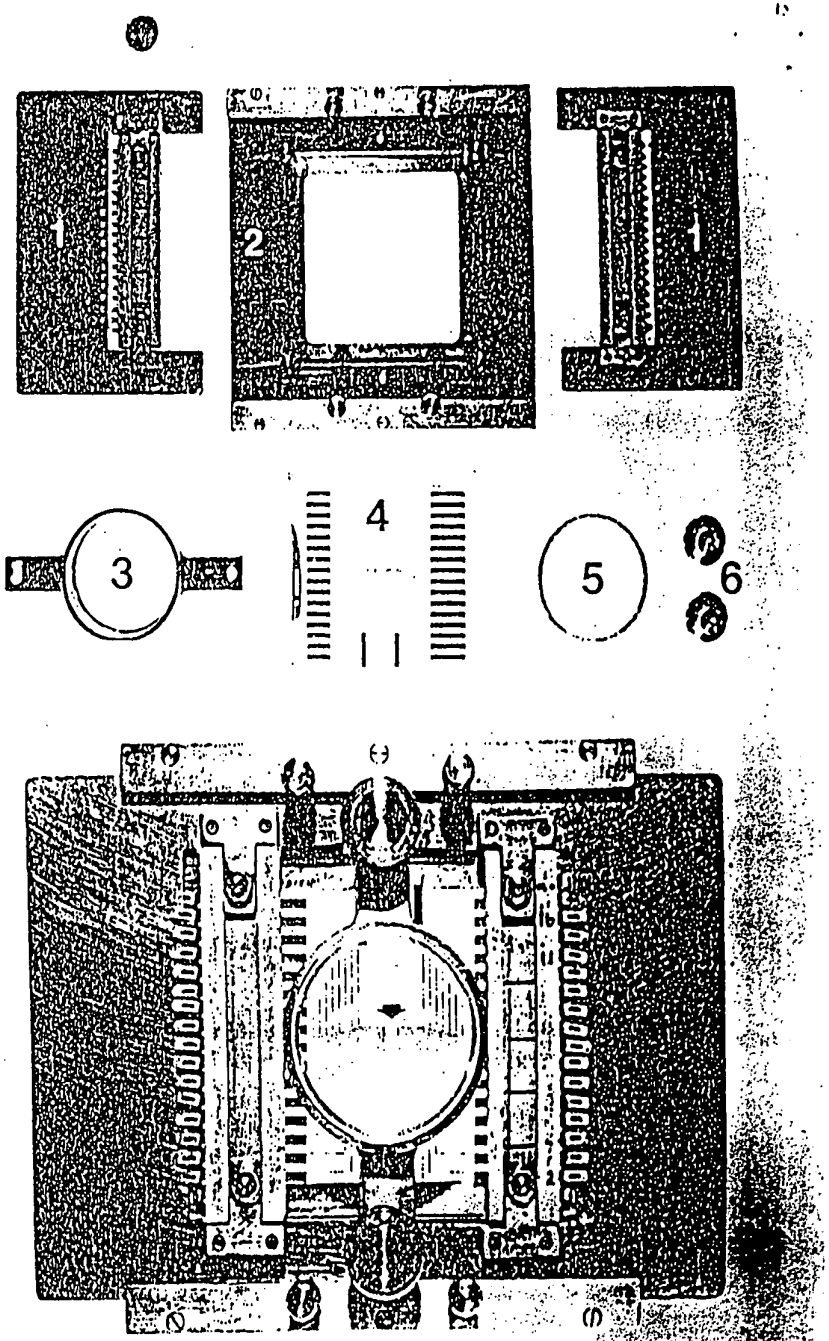


Fig. 1. Disassembled (top) and assembled (bottom) culture and recording chamber. The floor of the chamber is a glass multimicroelectrode plate containing 36 gold photoetched electrodes. The arrow points to the recording matrix shown with cultured cells in Fig. 2. 1, contact strips; 2, electrode plate holder; 3,

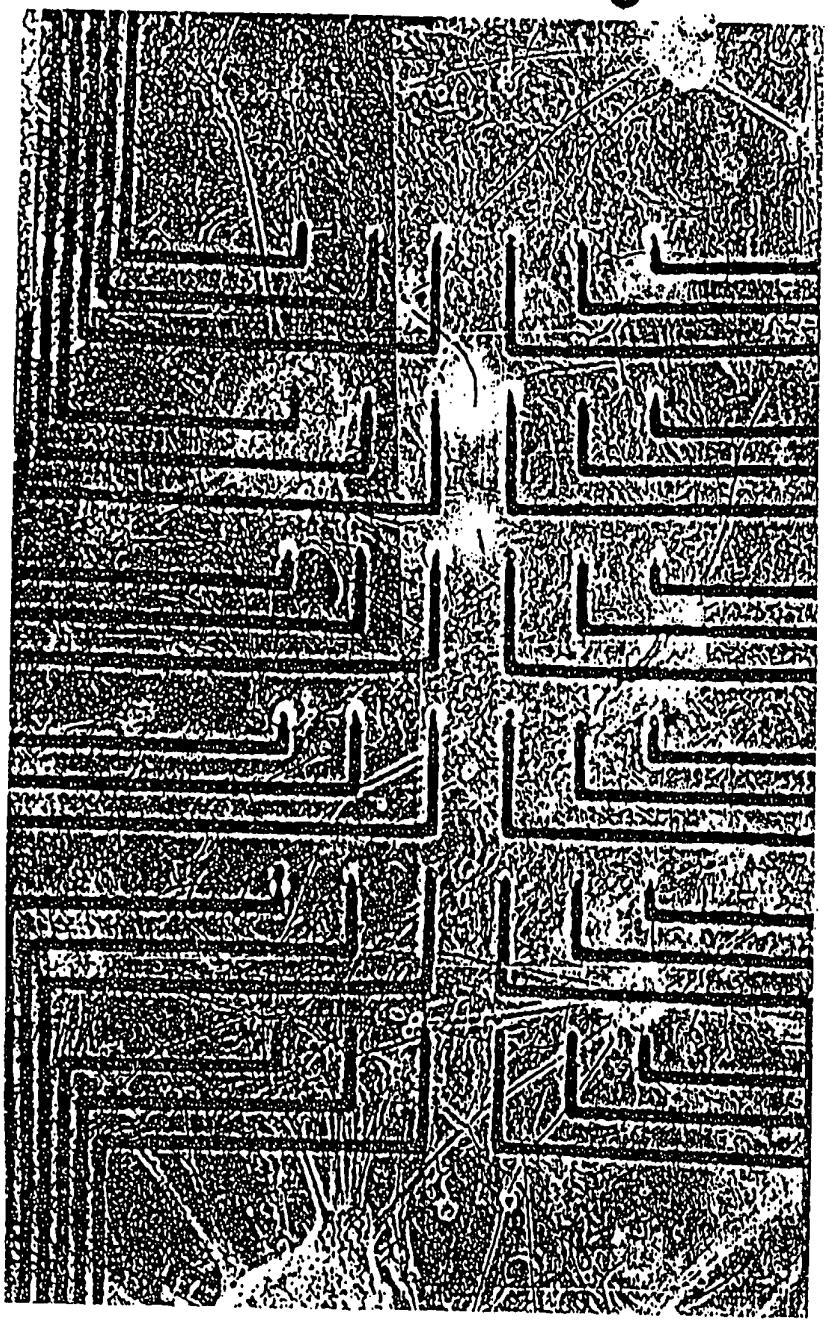


Fig. 2. Inverted microscope view of recording matrix showing the 36 microelectrodes (12 μm wide) and a variety of neuronal aggregates, small tissue fragments as well as a developing glia carpet 10 days after plating dissociated embryonal spinal cord segments on the multielectrode surface. The electrode tips are

Arrangements for electrophysiological recording

Prior to recording, the silicone gasket was replaced by a stainless-steel ring 28 mm in diameter that served as the chamber wall and ground electrode (see Fig. 1). Standard arrangements for signal amplification and oscilloscope display were used. During recording the culture medium was maintained at the proper pH and oxygen tension by a 20 ml/min moist flow of 5% CO_2, 35% O_2 and 60% N_2. Temperature within the recording chamber was maintained between 35 and 36°C except when deliberately varied with a microscope stage heater (Reichert) connected to a DC power supply.

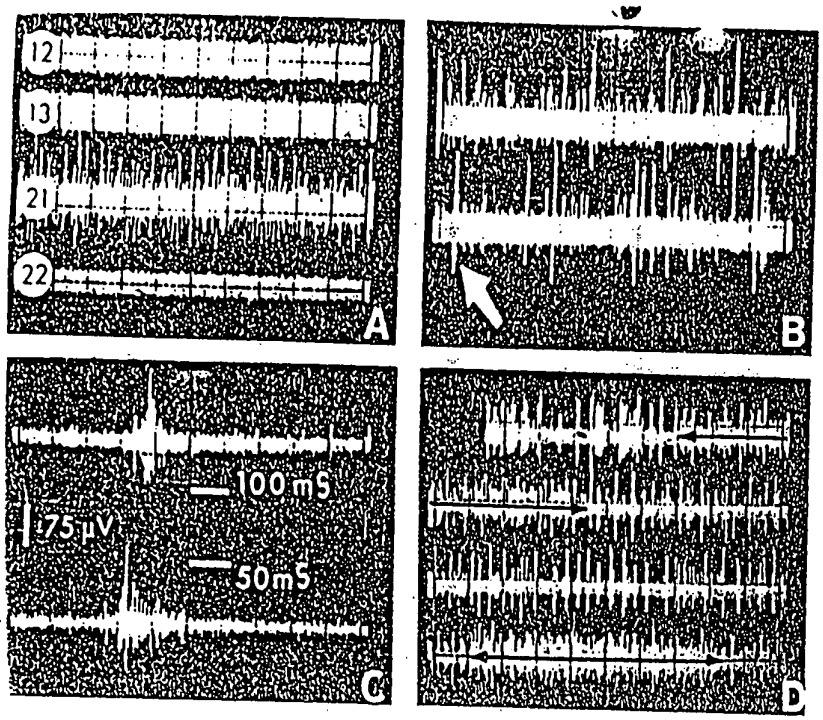
Results

Fig. 2 shows an inverted microscope view of mouse spinal tissue from 13-day embryos 10 days after seeding. Recordings were obtained from the clump of cells overlying the first electrode from the left in the second row (designated E21) which had an impedance of 7 MΩ.

Although Fig. 2 shows few identifiable neuronal profiles, the large number of fine processes originating from cell clumps or aggregates is indicative of a healthy culture. The tissue fragment centered on E21 is unfortunately too dense t allow identification of neurons. However, the diffraction patterns discernible within the fragment reveal most cell bodies to be below 15 μm diameter. Glial cells are well established in most regions of the recording matrix but have been prevented from overgrowing the culture by FdU added two days after seeding.

Representative extracellular electrophysiological recordings are shown in Fig. 3. All recordings shown were obtained from electrode 21 of Fig. 2 over a 48 h period. It can be seen that the electrode is monitoring complex spontaneous activity from several cells. No such activity was seen by any other electrode of the matrix. It is interesting that E22, situated only 20 μm from the edge of the active tissue fragment, is not monitoring any spike potentials (Fig. 3A). The microfragment exhibited a variety of activities ranging from rhythmic multi-unit bursts (Fig. 3B and C) to long periods of intensive multi-cell firing (Fig. 3D) during which time the bursting usually ceased. The largest spikes recorded were 200 μV in amplitude.

Fig. 4 depicts. spontaneous activity recorded from a thin sheet of spinal cell aggregates (12-day culture from 15-day mouse embryos). A photograph of this cell layer in the area of the recording electrode is shown in Fig. 5. In this case the activity was not complex and represented the regular firing of one or two single units. Changes in the frequency of spiking in response to a gradual lowering of the temperature over a period of 1 h as well as to the addition of sodium citrate (in a 25 mM concentration) demonstrated that this activity was biological in nature and not an artifact of the recording system. Fig. 4A and B shows activity at 34°C and 20°C respectively. During the period of cooling, spike frequency decreased from 17 to less than 2 spikes/10 s interval. Return to 34°C (Fig. 4C) resulted in total recovery of activity but with some loss of spike amplitude. These changes in firing frequency are depicted graphically in Fig. 6. Fig. 4D shows the effect of sodium citrate addition



e 3. Spontaneous extracellular activity recorded from 150 µm tissue fragment situated over the ctrode in row 2 and column 1 (denoted E21) of the recording matrix shown in Fig. 2. Oscilloscope es are sequential and do not represent simultaneous recordings. A: comparison of activity on adjacent ctrodes in rows 1 and 2 on the left half of the recording matrix. Calibration: 1 s/div, 40 µV/div. B: al activity with maximum signal amplitudes of 190 µV and noise levels of 30 µV. Calibration: div, 40 µV/div. C: multi-unit bursts that appear as single spikes in B (at arrow). D: slow sweep div) showing changes from bursting to long periods of intensive multi-unit activity (horizontal ss)

ich resulted in an increase of bursting, a decay of spike amplitude and finally the eversible termination of activity.

scussion

These recordings represent the first monitoring of activity with photoetched ctrodes from mammalian spinal tissue in culture. Furthermore, the observations e limited to 2 days for technical reasons or by the deliberate termination of tures to verify the bioelectric origin of the signals. Minor modification in cedures could easily expand the recording period. Recordings have also been ained from dissociated mouse brain tissue; however, on these occasions the al-to-noise ratios did not exceed 1.8 : 1. The increase in signal-to-noise ratios seen

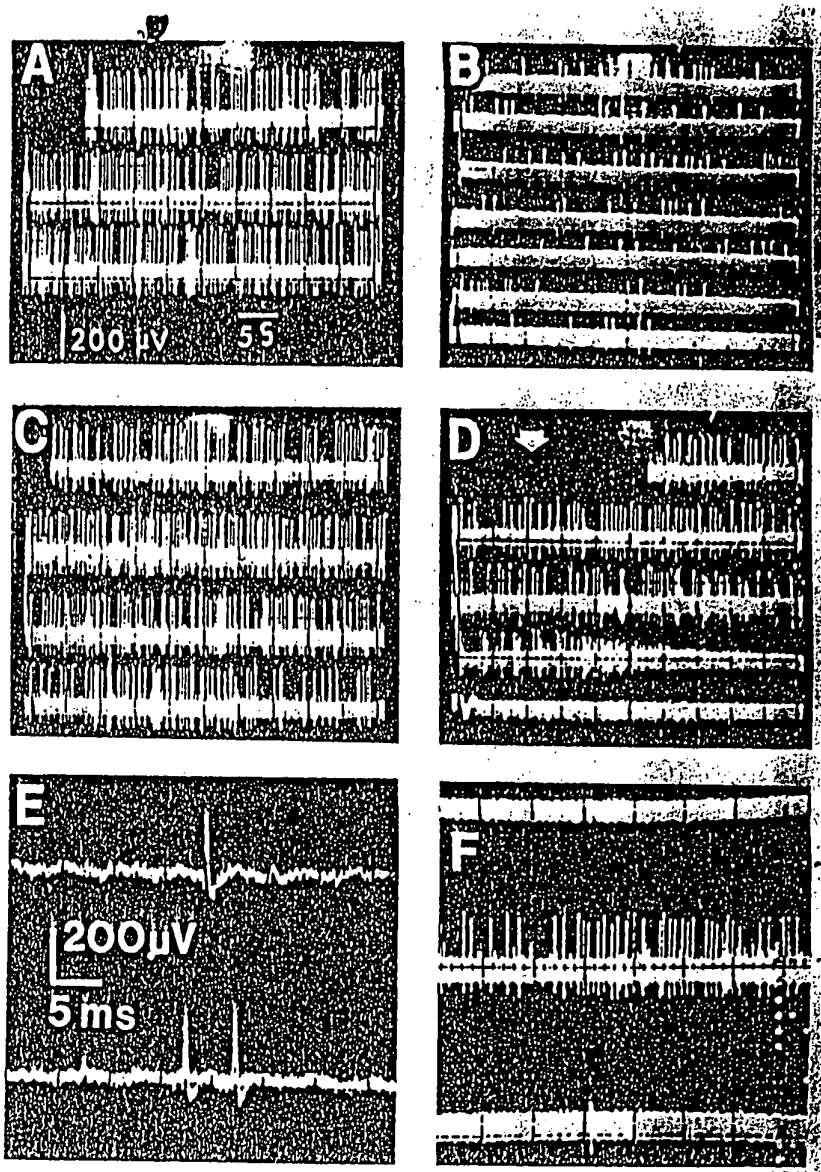


Fig. 4. Spontaneous extracellular activity from a thin sheet of aggregated spinal cells 12 days after dissociation and seeding. Recordings were obtained with the photoetched electrode identified in Fig. 5 (impedance: 4 MΩ). Calibration: A–D sweep 5 s/div; amplitude: 195 µV/div; band width: 100–10,000 Hz. Display resulting from sequential traces on storage oscilloscope. A: single-unit activity at 34°C. B: activity on same electrode 1 h later with temperature lowered to 20°C. C: recovery of activity with temperature restoration to 34°C over a 30 min period. D: reaction of active unit to addition of citrat. (at arrow). Note rhythmic bursting after a 110 s interval followed by a decrease in spike amplitude and complete cessation of activity. E: rapid sweeps depicting single action potentials (band width: DC 10,000 Hz plus 60 Hz filter) with maximum amplitudes of 360 µV and a signal-to-noise ratio of 8 : 1. F: signals from 3 adjacent electrodes located under the thin tissue sheet shown in Fig. 5. Only the center electrode re

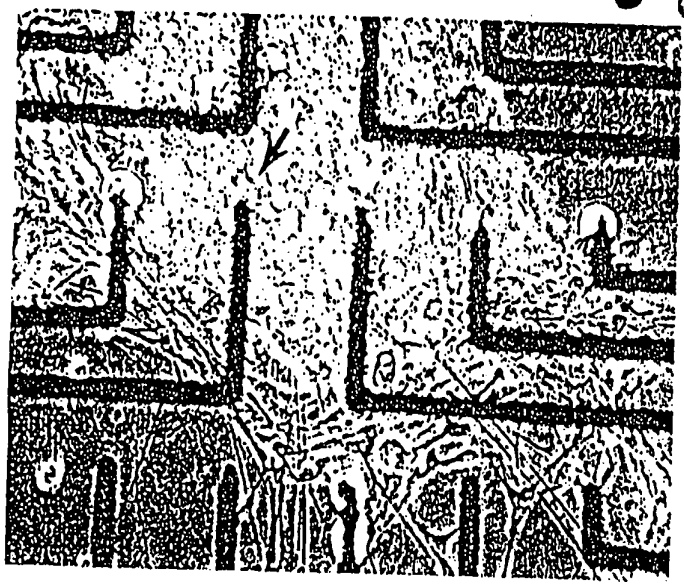


Fig. 5. Inverted microscope view of region producing activity depicted in Fig. 4. The tissue is almost a monolayer formed by reaggregated mouse spinal cord cells. The active unit, however, could not be identified. The arrow points to electrode with which data in Fig. 4 was obtained. Electrodes to either side did not monitor activity.

recently may be due to the introduction of the surface oxidation of the insulation layer which has resulted in improved cell-to-substrate adhesion. The usefulness of this as a general technique for promoting cell adhesion as compared to other methods of surface modification (collagen, polylysine, etc.) is currently under investigation in this laboratory and will be reported in a separate paper.

Although the characteristics of the activity shown in Figs. 3 and 4 would clearly

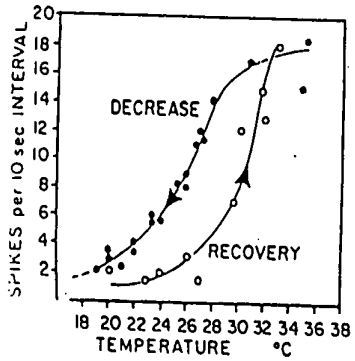


Fig. 6. Temperature dependence of spontaneous activity recorded from neuron described in Figs. 4 and 5. Temperature was lowered at 0.3°C/min and raised at 0.5°C/min.

be accepted as neuronal if recorded with standard metal electrodes, the introduction of a new recording technique demands greater caution. We have, therefore, attempted to demonstrate the biological origin of the signals with simple physical and chemical manipulations as already described in the Results section. The temporary increase in activity together with a decrease in spike amplitude and finally cessation of firing is a typical reaction to high concentrations of sodium citrate, a known calcium ion chelator. The temperature dependence of the spontaneous firing frequency and the spike amplitude is also typical of neurons (Gähwiler et al., 1972; Leiman and Seil, 1973). Most cells stop firing below 20°C, although not all cells display a temperature-dependent spontaneous firing pattern between 25 and 38°C. As far as recording system noise is concerned, none of the major categories (white noise, current or 1/f noise, and shot noise) are known to display sigmoidal dependence on temperature (Neher, 1974; DeFelice, 1981).

We have achieved satisfactory signal-to-noise ratios without the use of platinum black which, up to now, has been generally utilized as a means lowering electrode impedances (Thomas et al., 1972; Pine, 1980). In view of the inherent instability of such electrode tips (Gesteland et al., 1959; Robinson, 1968), the use of platinum black should be avoided if the long-term monitoring of signals is a major objective.

The multielectrode plates used in these experiments are obviously designed for the long-term, simultaneous recording of spike activity from cultured neuronal monolayers in which the active units and most of their interconnections can be identified and observed. Furthermore, we are developing laser microbeam cell surgery techniques that will allow the simplification of the monolayer or a change in neuronal interconnections during recording (Higgins et al., 1980). In this paper we have taken the initial electrophysiological step by demonstrating that mammalian CNS tissue can be satisfactorily coupled to a polysiloxane insulation layer and that good signal-to-noise ratios can be obtained. Although preliminary data indicate that healthy-appearing cultures can be maintained on the polysiloxane for over 3 weeks, we cannot yet make any statements about signal acquisition during that period of time. Monolayers and presumably the surfaces of tissue microfragments are not morphologically stable. Glial movement and elastic readjustments will probably cause a shifting of active units relative to the recording crater. The reinsulation of these electrode craters is also a problem that may be decreasing the probability of simultaneous recording and to which the FdU treatment appears to be only a partial solution. Nevertheless, the advantages of recording with multimicroelectrode surfaces in culture are substantial and justify a serious attack on these problems. There is no other approach that will give us the capability of long-term, continuous monitoring, stimulation and observation of over 30 neurons in a controlled chemical environment. Neither the conventional methods of recording nor the utilization of voltage-sensitive dyes offer such a combination of advantages.

Acknowledgements

The authors wish to thank Mr. Andy Pratt for his technical assistance. We also thank the Siemens Corporation of Munich, Germany for the gratuitous production

of the photoetched plates in 1976. Some plates can still be utilized after 4 years of experimentation. We acknowledge the assistance of the Sandoz Corporation of Basel, Switzerland and specifically thank Dr. Meier-Ruge for making available a UV laser microbeam system.

Finally, we are grateful for the critical support from NINCDS through NIH Grant 5R01 NS 15167

References

DeFelice, L.J. (1981) Introduction to Membrane Noise. Plenum Press, N.Y., chapter 5.

Gähwiler, B.H., Mamoon, A.M., Schlapfer, W.T., and Tobias, C.A. (1972) Effects of temperature on spontaneous bioelectric activity of cultured nerve cells, Brain Res., 40:527–533.

Gesteland, R.C., Howland, B., Lettvin, J.Y. and Pitts, W.M. (1959) Comments on microelectrodes, Proc. IRE, 47:1856–1862.

Gross, G.W. (1979) Simultaneous single unit recording in vitro with photoetched, laser deinsulated gold multi-microelectrode surface, IEEE Transact. Biomed. Engng, BME 26:273–279.

Gross, G.W., Rieske, E., Kreutzberg, G.W., and Meyer, A. (1977) A new fixed array multimicroelectrode system designed for long term monitoring of extracellular single unit neuronal activity in vitro, Neurosci. Lett., 6:101–105.

Higgins, M.L., Smith, M.N. and Gross, G.W. (1980) Selective cell destruction and precise neurite transection in neuroblastoma cultures with pulsed UV laser microbeam irradiation: an analysis of mechanisms and transection reliability with light and scaning electron microscopy, J. Neurosci. Meth., 3:83–99.

Leiman, A.L. and Seil, F.J. (1973) Spontaneous and evoked bioelectric activity in organized cerebellar tissue culture, Exp. Neurol., 40:748–758.

Neher, E. (1974) Elektronische Messtechnik in der Physiologie, Springer, Berlin, pp. 53–55.

Pine, J. (1980) Recording action potentials from cultured neurons with extracellular microcircuit electrodes, J. neurosci. Meth., 2:19–31.

Ransom, B. R., Neale, E., Henkart, M., Bullock, P.N. and Nelson, P.G. (1977) Mouse spinal cord in cell culture I. Morphological and intrinsic neuronal electrophysiological properties, J. Neurophys., 40:1132–1150.

Robinson, D.A. (1968) The electrical properties of metal microelectrodes, Proc. IEEE, 56:1065–1071.

Thomas, C.A., Jr., Springer, P.A., Loeb, G.E., Berwald-Netter, Y. and Okun, C.M. (1972) A miniature microelectrode array to monitor the bioelectric activity of cultured cells, Exp. Cell Res., 74:61–66.

Journal of Neuroscience Methods, 5 (1982) 23–31
Elsevier Biomedical

A comparative study of EM fixation procedures for the adult rat spinal cord based on regional blood flow

Pamela K. Hill, Jack C. de la Torre, Susannah M. Thompson, Denise F. Bullock and Sheila Rosenfield-Wessells

Eastern Virginia Medical School, Department of Anatomy, Norfolk VA, and (J.C.T.) University of Miami School of Medicine, Department of Neurosurgery, Miami, Fl. (U.S.A.)

(Received October 10th, 1980)
(Revised version received May 30th, 1981)
(Accepted July 27th, 1981)

Key words: sodium cacodylate buffered perfusate — sodium phosphate buffer — spinal cord — rat

Adult rat spinal cords were perfused intracardially with aldehyde fixatives containing either sodium cacodylate or sodium phosphate buffer for EM studies. The perfusion flow rate of 24 cc/min for the first 3 min and 33 cc/min for completion of prefixation was found to be optimal. Ultrastructural evaluation of the L-1 fasciculus gracili, dorsal and ventral horns demonstrated that the quality of fixation is similar despite a two- to three-fold greater blood flow rate to the dorsal and ventral horns, respectively. Sodium cacodylate buffered perfusate provides superior clarity of organelle membranes, myelin lamellae and axoplasmic constituents when compared with sodium phosphate buffer.

Introduction

Adequate fixation of mammalian spinal cord for ultrastructural studies is undoubtedly a difficult challenge in neuroanatomical research mainly because of the variable density and dimension of the intrinsic microvasculature. Peters (1970) contends that white matter requires greater perfusion pressure than does gray matter for maximum fixation. Even after that technical adjustment is made, the result may still be unsatisfactory since the same segment of tissue may have areas that are well preserved while others are poorly fixed (Lampert and Cressman, 1964). This study was conducted to examine the use of various perfusion flow rates and to compare sodium cacodylate with sodium phosphate buffer in aldehyde fixation using adult rat spinal cord. The specific objective was to achieve consistent, reliable preservation of the dorsal funiculi for ultrastructural examination since its diminutive microvasculature often hinders such success.

Neuroscience Vol. 7, No. 5, pp. 1243 to 1256, 1982
Printed in Great Britain

0306-4522/82/051243-13$03.00/0
Pergamon Press Ltd

MULTIPLE ACTIONS OF ACETYLCHOLINE ON HIPPOCAMPAL PYRAMIDAL CELLS IN ORGANOTYPIC EXPLANT CULTURES

B. H. GÄHWILER and J. J. DREIFUSS
Preclinical Research, Sandoz Ltd., 4002 Basle, Switzerland and
Department of Physiology, University of Geneva, Medical School, 1211 Geneva, Switzerland

Abstract—Hippocampal cultures were prepared from 7- to 10-day-old rats by means of the roller-type technique. The preservation of the characteristic hippocampal cytoarchitecture allowed, after many weeks *in vitro*, impalement of pyramidal cells by microelectrodes under visual control. Application of 10^{-7} to 10^{-5} M acetylcholine to the bath depolarized hippocampal pyramidal cells, strongly increased their rate of firing and induced paroxysmal depolarization shifts. This depolarizing action was accompanied by a reduction in the amplitude of evoked postsynaptic potentials. Whereas it was not clear whether the decrease in the amplitude of the excitatory postsynaptic potentials was only a result of membrane depolarization, acetylcholine clearly and reversibly reduced the potency of evoked inhibitory postsynaptic potentials.

Iontophoresis of acetylcholine to the perisomatic region of pyramidal neurons, like acetylcholine applied to the bath, increased their firing rate and powerfully decreased the amplitude and duration of spontaneous and evoked inhibitory postsynaptic potentials. In contrast, iontophoresis of acetylcholine in the pyramidal cell layer at a distance from the recorded neuron generated a hyperpolarizing response associated with a reduction in firing rate. At high current strength, the initial hyperpolarization was (often) followed by a paroxysmal depolarization shift. High frequency electrical stimulation with electrodes located close to the acetylcholine pipette in the pyramidal cell layer (i.e. about 1 mm away from the recorded neuron) mimicked the acetylcholine effect.

Resistance measurements indicated that membrane input resistance was decreased in the majority of cells during application of acetylcholine. This decrease in membrane resistance may result from a direct action of acetylcholine or from an increased synaptic activity. Synaptic alterations induced by acetylcholine were quick in onset and in recovery, while the increase in the rate of firing occurred somewhat later. Atropine (10^{-5} M), which had no significant action by itself, completely abolished the action of acetylcholine applied to the bath or by iontophoresis. In contradistinction, naloxone did not influence the acetylcholine effects, although opiates and opioid peptides produce paroxysmal depolarization shifts in pyramidal cells which resemble those induced by acetylcholine.

Addition of 8–16 mM magnesium to the bathing solution or exposure of the cultures to a calcium-free solution containing 1 mM cobalt abolished the effects of acetylcholine. In the presence of 10^{-6} g/ml tetrodotoxin, 10^{-5} M acetylcholine decreased the membrane input resistance of pyramidal cells, reduced their threshold for the generation of tetrodotoxin-resistant spikes and generated paroxysmal depolarization shifts in a proportion of pyramidal cells. These results suggest that acetylcholine facilitates the generation of calcium-spikes and can initiate epileptiform depolarization shifts which appear to be intrinsic to pyramidal cells and which presumably result from a large influx of calcium-ions.

Various investigators have reported a slow muscarinic excitatory effect of iontophoretically-applied acetylcholine (ACh) on hippocampal pyramidal cells.[2,3,4,11,25,27,28] This action of ACh was postulated to be of postsynaptic origin and mediated by a decrease in potassium permeability,[1,15] analogous to the action of ACh reported for neurons of the cerebral cortex.[17]

Other studies showed that ACh also exerts a presynaptic inhibitory action in the hippocampus.[9,14,18,22,30,31] Moreover, ACh has been proposed to exert a neuromodulatory role in the hippocampus in view of the fact that ACh-induced increases in membrane input resistance and burst firing lasted for many hours.[1]

In this study, we have used organotypic explant cultures of rat hippocampus and we show that ACh effects on pyramidal cells are diverse and persist after long-term cholinergic deafferentation.

Abbreviations: ACh, acetylcholine; EPSP/IPSP, excitatory/inhibitory postsynaptic potential; GABA, γ-aminobutyrate; PDS, paroxysmal depolarization shift; TTX, tetrodotoxin.

EXPERIMENTAL PROCEDURES

Hippocampal cultures were prepared from 7–10-day old rats. Transverse slices, 350–450 μm thick, were cut with a McIlwain tissue sectioner, embedded in a plasma clot and cultured for 3–11 weeks by means of the roller-tube technique.[7] The cultures were fed at weekly intervals with a

medium consisting of horse serum (25%), Hanks' balanced salt solution (25%) and basal medium (Eagle) (50%), supplemented with glucose to a final concentration of 6.5 mg/ml.

Standard electrophysiological techniques were used for recording and for local application of ACh. Membrane input resistance was determined by applying negative current pulses of 120 ms duration and of 0.3 to 0.5 nA amplitude. Electrodes for intracellular recordings pulled from fine capillary tubing (external diameter 1 mm, internal, 0.75 mm) had a resistance of 50–80 MΩ and were filled with 3 M potassium acetate; glass electrodes used for monopolar field stimulation and for iontophoretic application of ACh had a tip diameter of 1–3 μm. They could be positioned independently of the recording electrode and contained either 1 M sodium chloride or 0.1 M ACh; leakage of drug was minimized by application of a −40 nA retaining current. ACh, as well as all other substances to be tested, were directly added to Hanks' balanced salt solution which also served as the control solution.

The following compounds were used: acetylcholine chloride (Sigma), atropine sulphate (Sigma), L-glutamate (Sigma), naloxone (Endo), tetrodotoxin (TTX, Sankyo).

RESULTS

Roller-tube cultures flatten during the first four weeks to such an extent that individual nerve cells can easily be viewed with phase contrast microscopy.[7] Despite this thinning, the characteristic hippocampal cytoarchitecture remained intact (Fig. 1). Activity was recorded intracellularly from a total of 44 pyramidal neurons. All cells had resting potentials of at least −50 mV and their membrane input resistance ranged between 40–95 MΩ. Most cells spontaneously displayed action potentials and produced sequences of excitatory (EPSP) and inhibitory (IPSP) postsynaptic potentials lasting up to a second in response to monopolar field stimulation of adjacent tissue. Stable recordings were obtained for hours so that the bathing solution could often be exchanged up to 50 times without interfering with the quality of recording.

Bath application of acetylcholine and glutamate

Bath applied ACh (10^{-7} to 10^{-4} M) depolarized hippocampal pyramidal cells and increased their rate of firing. Often sudden 'paroxysmal' depolarization shifts (PDS) were observed (Fig. 2B) which were accompanied by bursting discharges and followed by a marked membrane hyperpolarization (Fig. 2B). L-glutamate (10^{-4} to 10^{-3} M) also excited and depolarized pyramidal cells (Fig. 2A), but without causing the appearance of PDS. The actions of both ACh and L-glutamate were accompanied by a gradual increase in membrane noise and a concomitant decrease in the amplitude and duration of the evoked synaptic potentials. The interpretation of the effects on the evoked synaptic potentials is somewhat complicated by the increased membrane noise, but it is interesting to notice that no IPSPs could be detected during membrane depolarizations, whereas prominent EPSPs appeared during membrane hyperpolarizations (Fig. 2). Threshold concentration for the ACh effects was 10^{-7} M (dose-response tested in 6 cells).

Local, iontophoretic application of acetylcholine

We have taken advantage of the easy accessibility to pyramidal cells in roller-tube cultures in order to apply ACh iontophoretically in the immediate vicinity of the pyramidal cell soma from which activity was recorded intracellularly. ACh slightly depolarized the pyramidal cells and strongly increased their rate of firing (Fig. 3). The effect was, compared with that of L-glutamate (not shown), slow in onset and outlasted the period of drug ejection. In the majority of cells, perisomatic application of ACh resulted in a decrease of the membrane input resistance (Fig. 3B). Turning off the retaining current was usually sufficient to excite pyramidal cells; application of higher currents of ACh (up to 200 nA) strongly depolarized the cells and sometimes blocked their spike-generating mechanism (Fig. 7A).

In contrast, application of ACh into the pyramidal cell layer 0.5–1.5 mm away from the recorded cell resulted in a hyperpolarization and a reduction in firing rate (Fig. 4), but also caused a decrease in membrane input resistance (Fig. 4B and E). During the hyperpolarizations, the membrane potential appeared to be shunted at a level close to the reversal potential of the IPSPs. At higher iontophoretic current strength (Fig. 4C to E), the membrane also tended to hyperpolarize (Fig. 4C and D, but not in E), but these ACh-induced hyperpolarizations were at times followed by PDS with inactivated spike discharges (Fig. 4F, E and 8B).

This 'distant' effect of ACh could be mimicked by field stimulation. When an electrode was placed into the pyramidal cell layer at various distances from a pyramidal cell from which activity was simultaneously recorded, monopolar field stimulation elicted an EPSP-IPSP sequence (Fig. 5A). The response to repetitive stimuli depended on the intensity of the stimulation. At low intensity, IPSPs appeared to superimpose and the response consisted in a sustained hyperpolarization, whereas at higher stimulus strength (Fig. 5B), IPSPs disappeared, EPSPs of large amplitude and even PDS were generated.[12]

Effects on the inhibitory postsynaptic potentials

When ACh was ejected iontophoretically onto the recorded pyramidal cell soma or added to the bathing solution, spontaneous (Fig. 3) and evoked (Fig. 2B) IPSPs diminished in amplitude. In order to quantify this effect, the response to field stimulation was studied while the cell was briefly depolarized (depolarizing current pulse injected through recording electrode) in order to exaggerate the IPSPs and to induce a higher frequency of firing. Bath application of 10^{-6} M ACh (Fig. 6) markedly reduced the duration of the inhibitory pause in firing induced by field stimulation. The action of ACh was rapid in onset and completely

Fig. 1.
reveals

Fig. 1. Cytoarchitecture of hippocampal explant cultured for 49 days. Staining with toluidine blue reveals the preservation of the pyramidal cell layer (areas CA1 to CA3) as well as the granule cell layer of the dentate gyrus (DG). Scale, 0.3 mm.

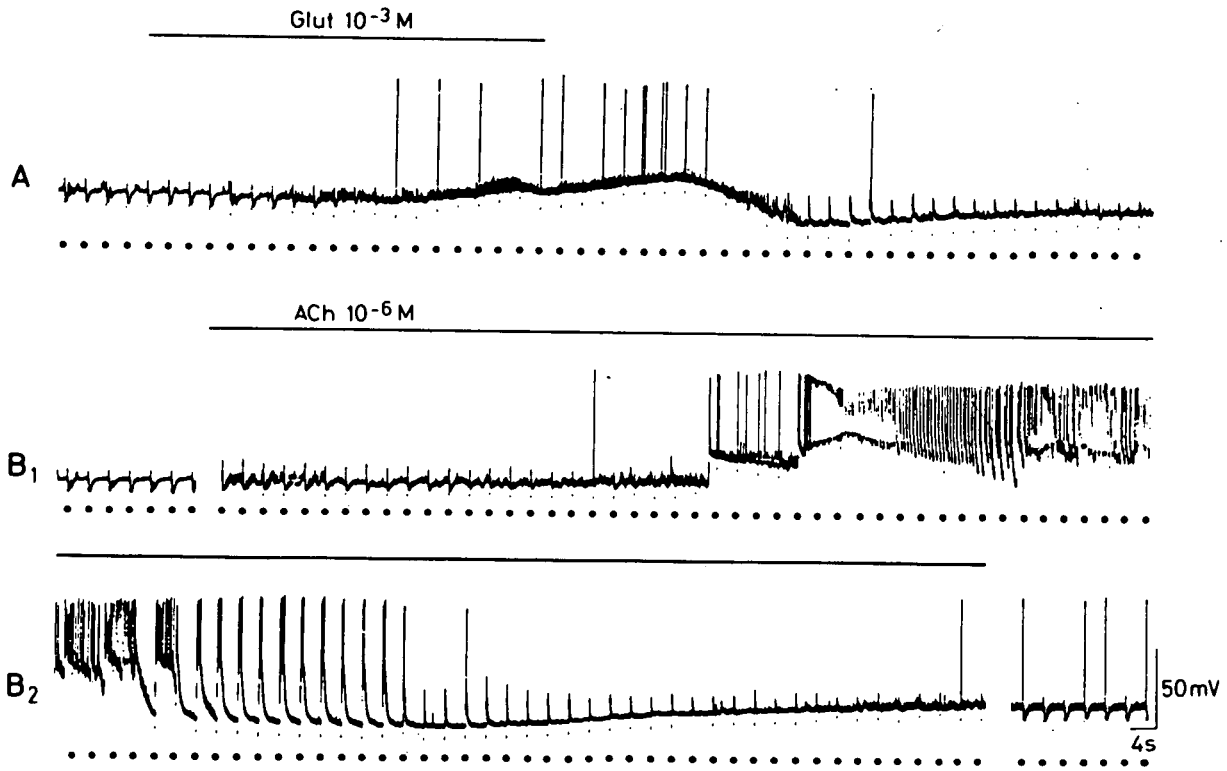


Fig. 2. Comparison of the effects of L-glutamate and acetylcholine applied to the bath on the activity of a hippocampal pyramidal cell. Monopolar field stimulation within the hippocampal explant (at times indicated by dots) elicited an EPSP-IPSP sequence. Stimulation parameters were 0.1 ms, 1/3 Hz, 6 μA. A. During application of 10^{-3} M L-glutamate, the evoked synaptic potentials gradually disappeared as the membrane potential became more noisy. The L-glutamate-induced depolarization was followed by a sustained membrane hyperpolarization during which field stimulation evoked EPSPs of large amplitude. B. Continuous record, except for the first and last seven evoked responses which were recorded 20 s before and 80 s after application of 10^{-6} M ACh, respectively. Like L-glutamate, ACh abolished the evoked synaptic responses, but in contrast to L-glutamate, ACh elicited a depolarization shift associated with bursting discharges. During the postburst hyperpolarization, EPSPs of large amplitude were evoked.

reversible. When ACh was applied iontophoretically, both onset and recovery of synaptic alterations often occurred more rapidly than the increase in the rate of firing.

Specificity of the acetylcholine effect

In order to address the question of specificity, the action of atropine on the effects induced by ACh was analyzed in 7 cells. Atropine at a concentration of 10^{-5} M had, by itself, no detectable effect on the bioelectric activity. The drug, however, completely prevented the action of ACh applied to the bath or by iontophoresis (Fig. 7). The excitatory effects of perisomatic as well as the inhibitory effects of distant application of ACh were all blocked by atropine. Naloxone (10^{-6} and 10^{-5} M), tested on 4 cells, produced neither an effect on its own nor influenced the response of pyramidal cells to ACh.

Effects on membrane input resistance in normal solution and in solutions containing Mg^{2+}, Co^{2+} or tetrodotoxin

Distant iontophoretic application of ACh in all cases decreased the membrane input resistance of the recorded pyramidal cell (Fig. 4B, 8B). Perisomatic iontophoresis (Fig. 3B, 9B) or bath application of ACh (Fig. 9A) led in 14 out of 18 pyramidal cells to a transient decrease in input resistance, although an increase in membrane input resistance was observed in the remaining 4 cells (Fig. 8A).

In normal bathing solution, resistance measurements are complicated by alterations in input resistance induced by synaptic potentials. In order to determine whether ACh exerts a postsynaptic effect, the cells were synaptically isolated, either by adding Mg^{2+} (8–16 mM, 4 cells tested) to the control solution or by exposing the cultures to a Ca^{2+}-free solution containing 1 mM Co^{2+} (10 cells tested). In cells which had been shown in control solution to respond (Fig. 9A, B), iontophoretic ACh had no effect on input resistance once these cells had been synaptically isolated (Fig. 9C). Similarly, even very high concentrations of ACh (up to 5×10^{-4} M) in the bathing solution influenced neither input resistance nor excitability of these decoupled cells (Fig. 9D).

Exposure of the cultures to a solution containing 10^{-6} g/ml TTX blocked spontaneous action potentials and synaptic activity in pyramidal cells, but spikes of longer duration, resembling the Ca-spikes described by Schwartzkroin & Slawski,[24] could still be evoked by depolarizing current pulses applied through the intracellular electrode. Under those conditions, bath application of 10^{-5} M ACh led to a reversible reduction in membrane resistance and, in 7

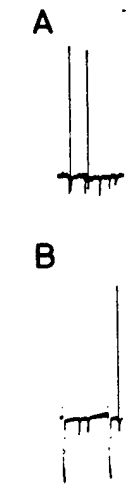


Fig. 3. Effect of perisomatic iontophoretic application of acetylcholine. Turning off the retaining current applied to the micropipette to prevent diffusion of ACh led to a prolonged increase in the rate of firing which was reversible, but clearly outlasted the duration of application of ACh. The amplitude of spontaneous IPSPs was decreased by ACh, but IPSPs recovered while the firing rate was still increased (A). In B, membrane input resistance (72 MΩ) was measured by application of negative current pulses of 120 ms duration and 0.4 nA amplitude. ACh slightly increased membrane conductance.

Fig. 4. Effects of distant application of acetylcholine on pyramidal cell activity. ACh was ejected iontophoretically into the pyramidal cell layer about 1 mm away from the recorded pyramidal cell. Low current strengths of ACh (0–40 nA) hyperpolarized pyramidal cells (A to C), reduced their membrane input resistance (B) and abolished evoked synaptic potentials (C). At higher current strengths of ACh (40 nA and more), depolarization shifts with bursting discharges were induced. These depolarizations were in some cells preceded by an initial hyperpolarization (D). Membrane input resistance was reduced prior to and during the depolarization shifts (E). Stimulus parameters for local field stimulation (C) were: 0.1 ms, 4 μA, 1/3 Hz. Resistance was measured with negative current pulses of 120 ms in duration and 0.4 nA (B) and 0.5 nA (E) in amplitude, applied at a frequency of 1/3 Hz.

Fig. 5. Mimicking the effect of acetylcholine by repetitive field stimulation. The monopolar stimulation electrode was placed into the pyramidal cell layer about 1 mm away from the recorded neuron. A single stimulus (indicated by star) elicited an EPSP-IPSP sequence. Using the same stimulus strength, repetitive stimulation at 10 Hz led to a marked membrane hyperpolarization (A), whereas at higher stimulus strength, the cell depolarized (B). Neither of these effects was influenced by application of 10^{-5} M atropine (not shown). Stimulus parameters were: 0.1 ms, 10 Hz, 4 μA (A) and 10 μA (B).

Fig. 6. Effect of acetylcholine on the potency of stimulus-induced inhibitory postsynaptic potentials. To exaggerate IPSPs, monopolar field stimulation was applied (arrow) during a depolarizing current pulse of 120 ms duration and 0.5 nA current strength applied through the recording electrode. During these depolarizing pulses, about 10–13 action potentials were evoked (not shown). In control solution, field stimulation elicited a short latency EPSP (triggering an action potential) followed by a long-lasting pause in firing. During application of 10^{-6} M ACh, the probability of firing during these IPSPs gradually increased, an effect which was rapid in onset and completely reversible. Examples of responses before (1), during (2, 3) and after (4) application of ACh are illustrated. The period of inhibition as indicated in the lower graph was defined as the interval between the time of onset of the IPSP and the first evoked spike. Since no spike was generated during the control period and after drug application, the interval between the onset of the IPSP and the end of the depolarizing pulse was taken as 100 ms and marked by open circles.

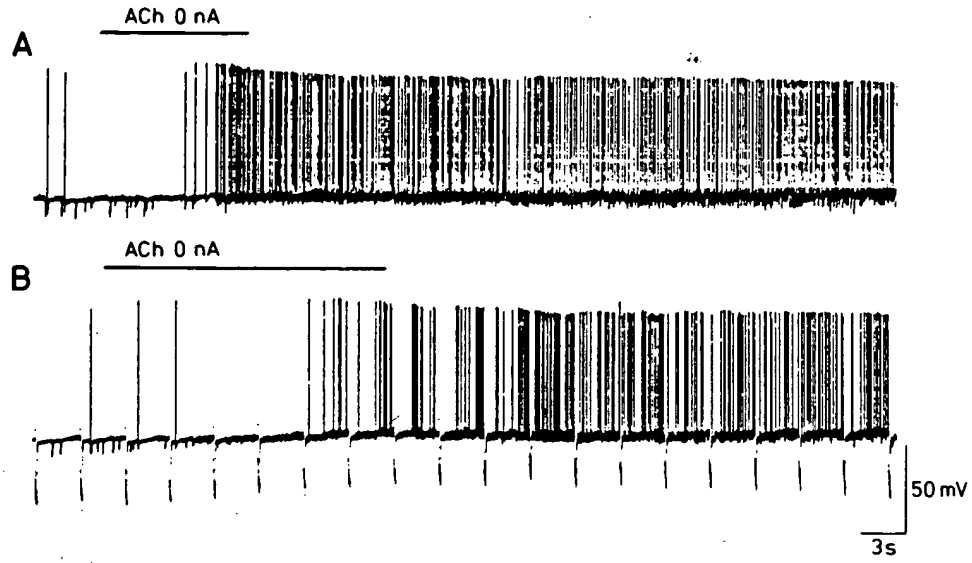

ACh 0 nA
A
ACh 0 nA
B
50 mV
3s

Fig. 3.

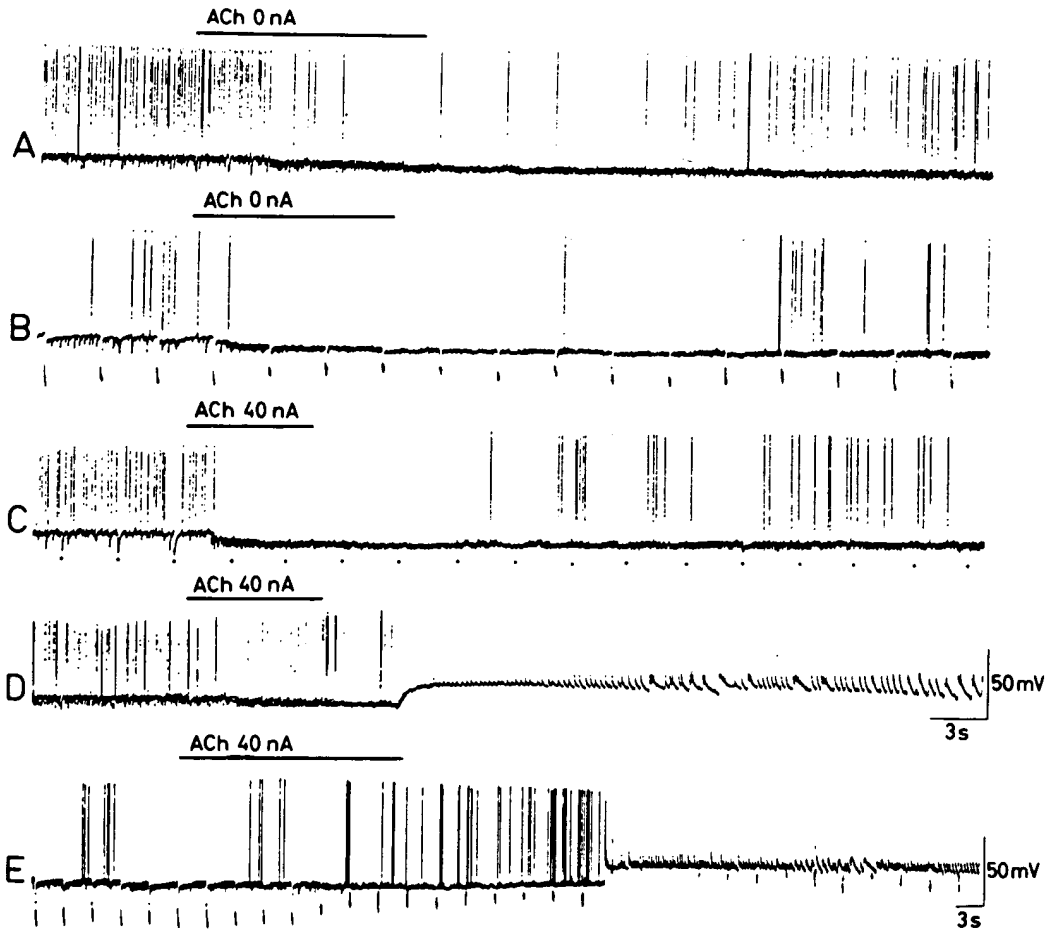

ACh 0 nA
A
ACh 0 nA
B
ACh 40 nA
C
ACh 40 nA
D
50mV
3s
ACh 40 nA
E
50mV
3s

Fig. 4.

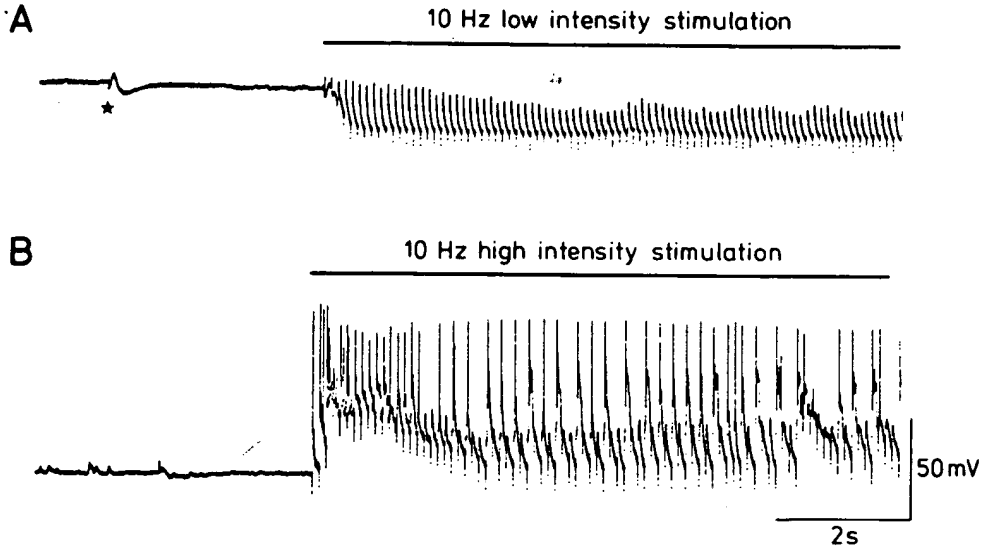


Fig. 5.

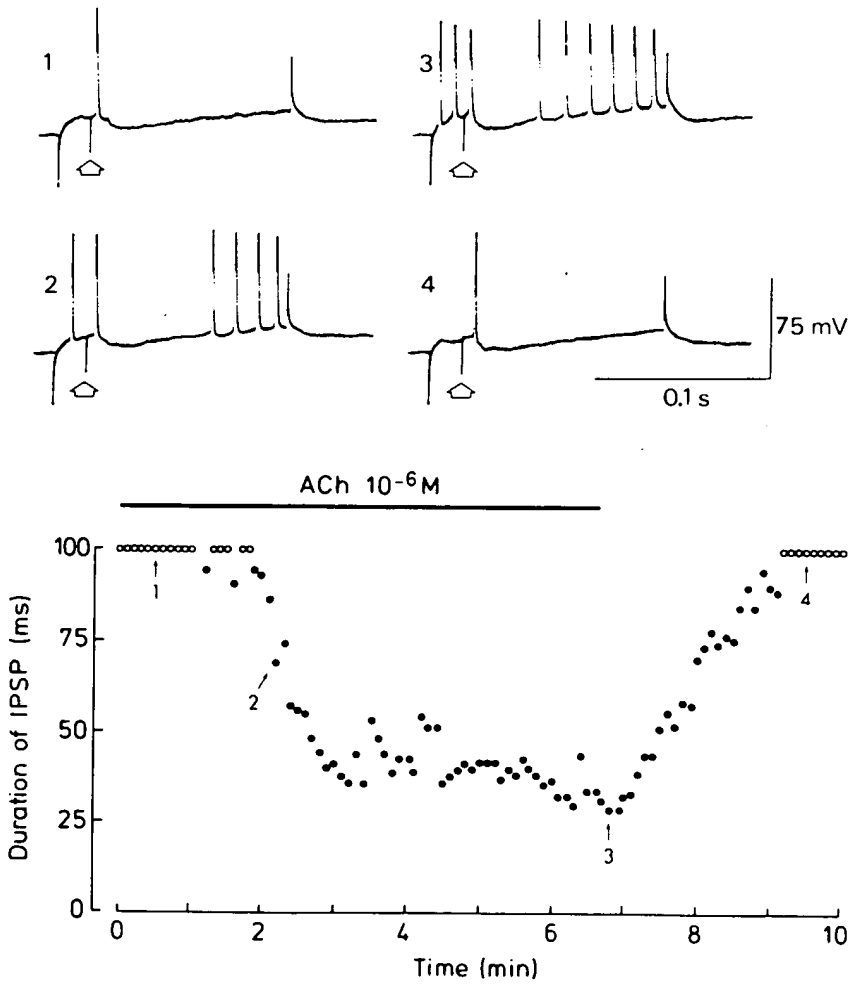


Fig. 6.

Fig. 7. Ef
depolariz
mechanis
mined by
prolonge

of 16 cells, to the
over, ACh clearly
ation of Ca-spik
contrast to ACh,
which in normal
detectable effect
these TTX-treate

ACh was found
dal cells in organ
First, ACh exert
second, produce
associated with a
firing. Third, loc
led, depending o
polarization or
brane. And fourt
which were syna
application of T
by application o
with the acute sli
trations of ACh e
notypic cultures.
difference is due
to development
ACh.

Postsynaptic acti

A presumably
ACh has been d

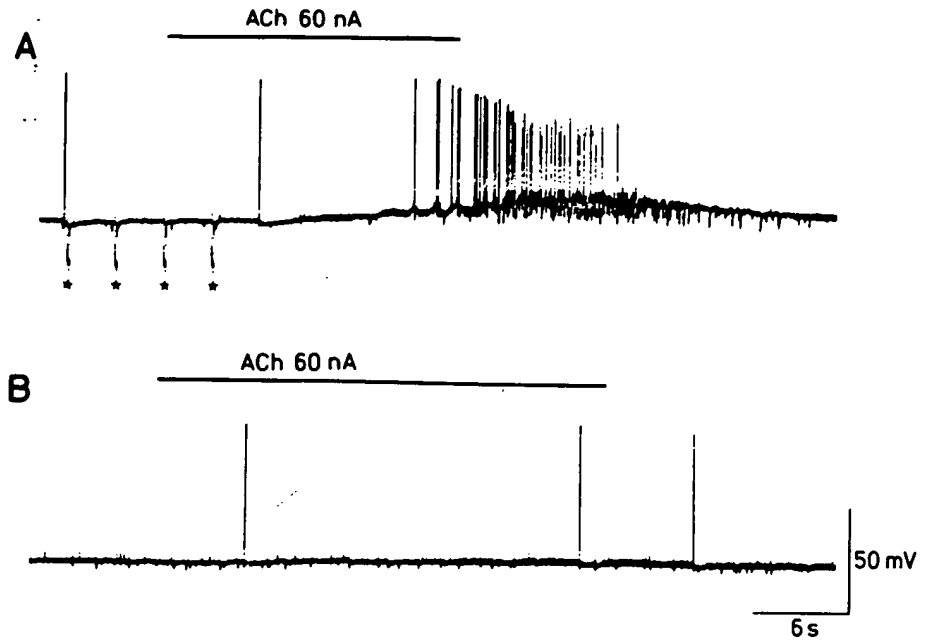


Fig. 7. Effect of atropine on the action of acetylcholine. Perisomatic iontophoretic application of ACh depolarized and excited the pyramidal cell (A). Due to the strong depolarization, the spike-generating mechanism was partially inactivated. Before drug application, membrane input resistance was determined by current pulses (indicated as stars). During application of 10^{-6} M atropine to the bath (B), even prolonged iontophoretic ejection of ACh was ineffective in exciting the pyramidal cell. The location of the iontophoretic electrode was identical during the recordings illustrated in A and B.

of 16 cells, to the generation of PDS (Fig. 10A). Moreover, ACh clearly reduced the threshold for the generation of Ca-spikes in 13 of 16 cells (Fig. 10D). In contrast to ACh, the enkephalin analogue FK 33-824 which in normal solution also induces PDS,[6] had no detectable effect on the activity of pyramidal cells in these TTX-treated cultures.

DISCUSSION

ACh was found to exert multiple effects on pyramidal cells in organotypic cultures of rat hippocampus. First, ACh exerted powerful effects on IPSPs and second, produced depolarizing responses which were associated with a slow or explosive increase in rate of firing. Third, local iontophoretic application of ACh led, depending on the ejection site, either to a depolarization or to a hyperpolarization of the membrane. And fourth, ACh depolarized pyramidal cells which were synaptically isolated from other cells by application of TTX, but did not affect those isolated by application of Co^{2+}- or Mg^{2+}-ions. Compared with the acute slice preparation, much lower concentrations of ACh excited hippocampal neurons in organotypic cultures. It is, however, not clear whether this difference is due to a more rapid diffusion of ACh or to development of denervation supersensitivity to ACh.

Postsynaptic actions of acetylcholine

A presumably postsynaptic muscarinic action of ACh has been described to cause a slow, prolonged depolarization associated with an increase in firing rate in hippocampal pyramidal cells.[2,3,4,25] This depolarization was accompanied[5,15] or followed[1] by an increase in membrane input resistance which was interpreted to be caused either by a reduction in resting potassium conductance[5] or by enhanced anomalous rectification.[1] Only very slight or no effects on input resistance were observed when ACh was iontophoretically applied to the perisomatic[30] or dendritic region of pyramidal cells.[14] In the present study, increases or decreases in input resistance of pyramidal cells were detected in response to application of ACh, with reductions clearly predominating. No effect of ACh was observed when Ca^{2+}-permeability was blocked by addition of Mg^{2+}- or Co^{2+}-ions, but ACh still caused a membrane depolarization and a decrease in input resistance in TTX-treated cultures. These results suggest that cultured pyramidal cells possess ACh receptors and that activation of these receptors leads to the generation of a depolarizing current which is presumably mainly carried by Ca^{2+}-ions.

Iontophoretic application of acetylcholine

An unexpected finding was that the response to local iontophoretic application of ACh depended on the site of ejection. Perisomatic application evoked a membrane depolarization and reduced the amplitude and duration of IPSPs. In contrast, more distant applications of ACh hyperpolarized pyramidal cells. At higher iontophoretic currents, paroxysmal depolariz-

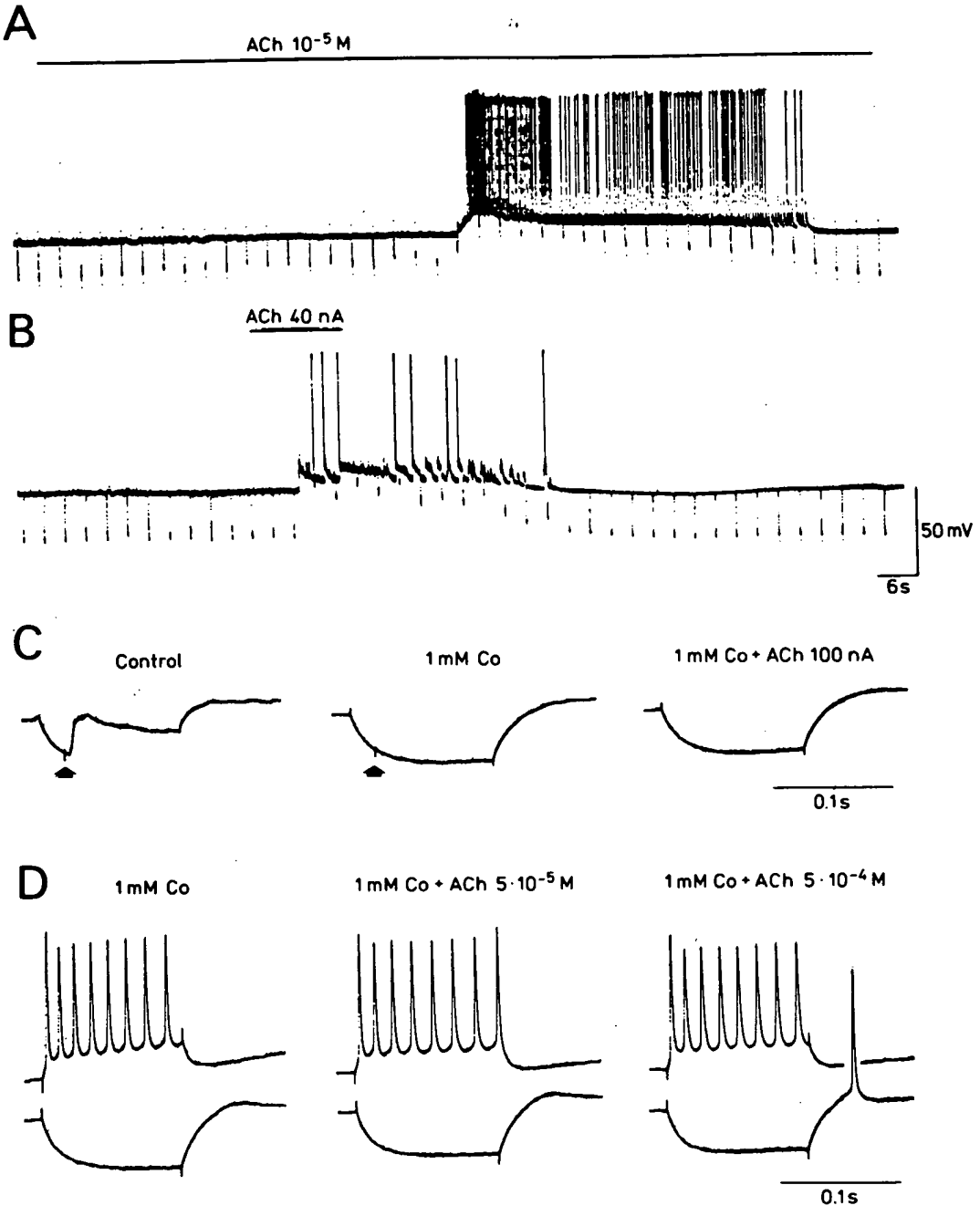


Fig. 9. Effect of acetylcholine on a synaptically-isolated pyramidal cell. ACh was shown to excite the pyramidal cell when the drug was applied to the bath (A) or by iontophoresis (B). Membrane input resistance was monitored throughout the experiment. Successful synaptic decoupling of pyramidal cell was ascertained by recording the effect of field stimulation (arrows) before and during exposure to a Ca^{2+}-free solution containing 1 mM Co^{2+} (C). Following synaptic isolation, ACh applied iontophoretically with a current of 100 nA to the perisomatic region of the cell (C) or applied to the bath at concentrations up to 5×10^{-5} M (D) had no detectable effect on either resting membrane potential or on membrane input resistance and membrane excitability as measured by intracellular current pulses (120 ms, ± 0.5 nA). At 5×10^{-4} M ACh, an anodal break response was evoked.

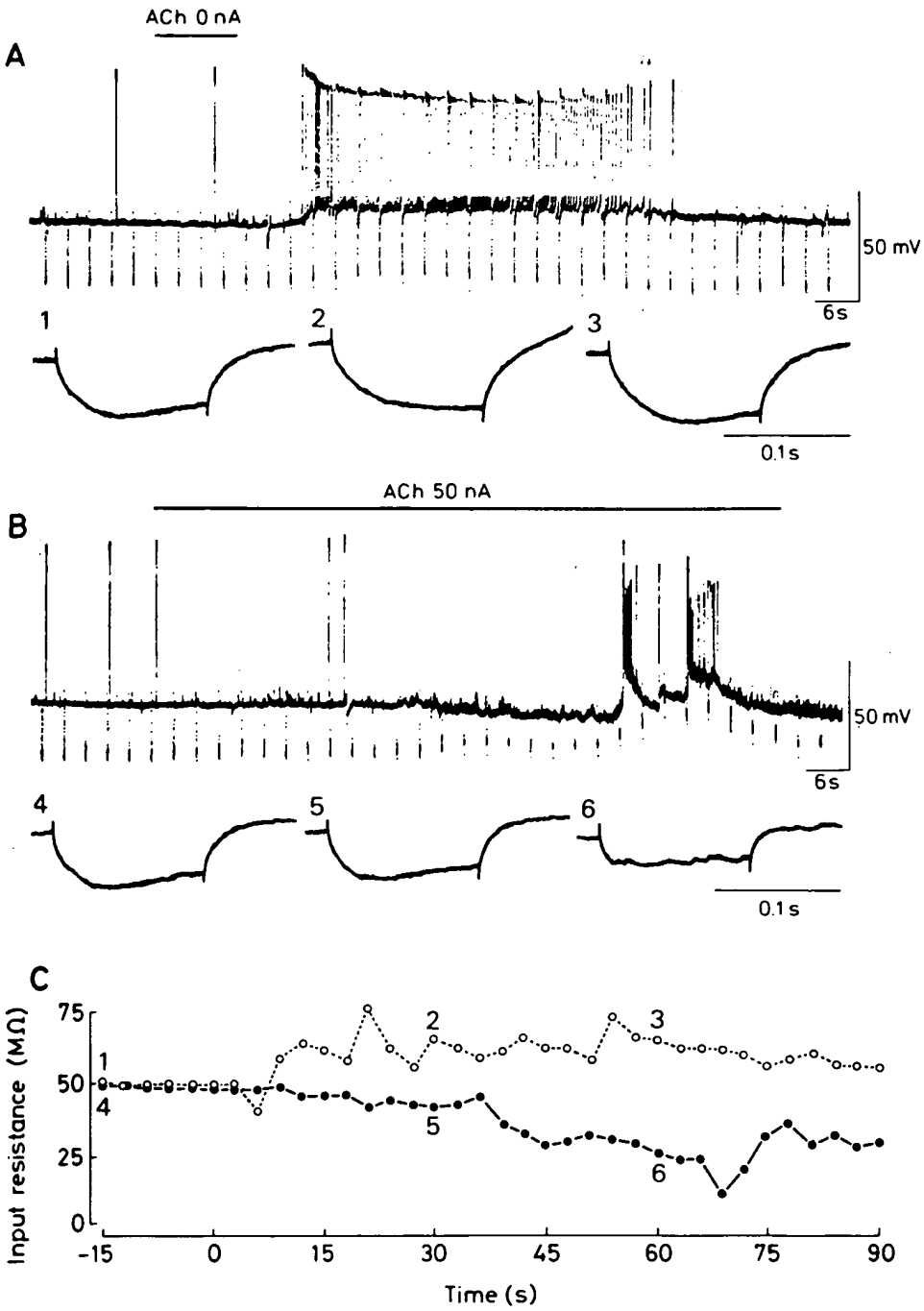


Fig. 8. Comparing the effect of perisomatic and of distant application of acetylcholine on membrane input resistance of a single pyramidal cell. Perisomatic application of ACh excited the pyramidal cell (A), whereas administration of ACh in the pyramidal cell layer at a distance of approximately 1 mm from the recorded neuron caused a biphasic response consisting in an initial hyperpolarization followed by a sudden depolarization with bursting discharges (B). Input resistance was measured every 3 s by applying negative current pulses of 120 ms duration and 0.5 nA amplitude. In C, the changes in input resistance observed in A (open circles) and in B (black dots) are illustrated graphically. Perisomatic ACh application led to a reversible increase, distant application to a reversible decrease in membrane input resistance. Examples of individual responses obtained during perisomatic (1–3) or distant (4–6) ACh applications, sampled at the times indicated in C, are shown under the records A and B.

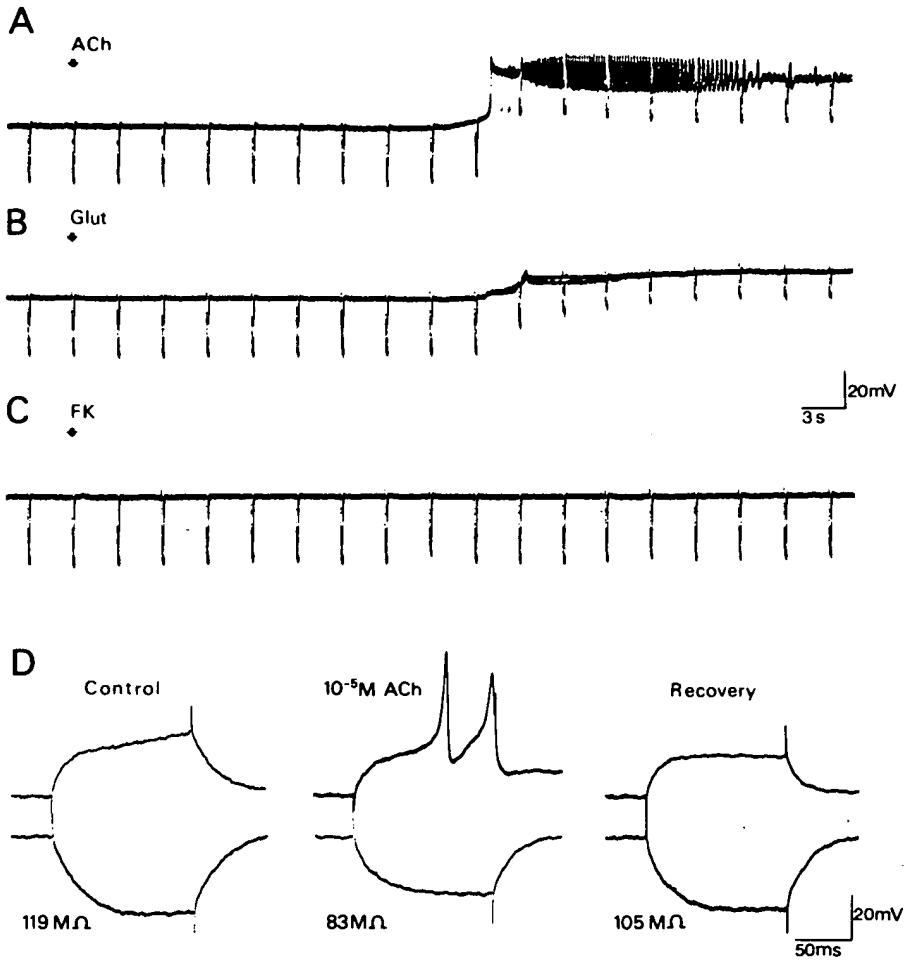


Fig. 10. Effect of acetylcholine on hippocampal pyramidal cells in the presence of tetrodotoxin. During exposure of the cultures to 10^{-6} g/ml TTX, ACh (10^{-5} M) induced a PDS which was characterized by sudden depolarizations with plateau-formation during which multiple spikes of long duration were generated (A). The depolarization was accompanied by a reduction in membrane input resistance. L-glutamate (10^{-3} M) also depolarized the pyramidal cell, but did not induce spiking activity (B). The opioid peptide FK 33-824 (FK), which in control solution is known to produce PDS, had no effect in the presence of TTX (C). Resistance was monitored throughout the experiment by intracellular current pulses (120 ms, −0.5 nA, 1/3 Hz). The recordings A to C were taken from the same cell. Following each drug application, the preparation was bathed in Hanks' balanced salt solution containing TTX until the membrane characteristics were similar to those seen before drug application. After prolonged application of TTX, fast Na-spikes and synaptic transmission (determined by measuring the response to field stimulation) reappeared within approximately 30 min of return to the control solution (not shown). D illustrates recordings from another cell which (in the presence of tetrodotoxin) was depolarized by acetylcholine without generation of paroxysmal depolarization shift. In such cases, it was possible to assess excitability and resistance changes directly induced by ACh. In this cell, 10^{-5} M ACh reversibly reduced the threshold for the generation of long duration spikes (presumably Ca-spikes) and decreased membrane input resistance. Examples of such responses are illustrated in D, in which the numbers indicate the calculated membrane input resistance (average of 8 responses). Parameters of the pulses were: 120 ms, 1/3 Hz, ±0.3 nA.

ation shifts were produced. Both the excitatory and the inhibitory effects of distant application of ACh were mimicked by electrical stimulation (Fig. 5). It appears, therefore, reasonable to assume that strong stimulation by ACh of cells located in the vicinity of the site of ejection gave rise to the inhibitory and excitatory responses. These effects could be mediated by local circuit interneurons or by inhibitory[16] as well as excitatory interactions[19] between pyramidal cells. Local effects of ACh could perhaps help to explain the biphasic hyperpolarization-depolarization sequence reported by Benardo & Prince,[1] following microdrop application of ACh to hippocampal slices.

Presynaptic effects of acetylcholine

A presumed presynaptic action of ACh on hippocampal neurons was first demonstrated by Yamamoto & Kawai[31] who reported that ACh abolished synapti-

cally-evoked gra
slices. Later stu
showing that AC
increased and p
application had
population spik
cations, ACh ha
disinhibitory effe
pal slices.[30] The
hypothesis that
pocampus is me
potency of IPS
was either appl
ejected onto th
neuron. Postsyn
brane input res
of GABA recep
The amplitude
was again not c
membrane dep
aptic effect of
shown also by
presynaptic ac
course than th

Paroxysomal d

During bath
phoretic adm
strength, pyra
depolarization
charges (see F
activity was n
of L-glutamate
and depolariz
ation shifts, h

1. Benardo L.
211, 227-2
2. Bird S. J. &
micro-ionto
3. Biscoe T. J
Lond. **183,**
4. Bland B. H
Can. J. Ph
5. Dodd J., D
guinea-pig
6. Gähwiler
Brain Res.
7. Gähwiler
8. Gähwiler
campus. *R*
9. Haas H. L
10. Haas H. L
Neuroscier
11. Herrling P
reaction m
12. Herrling P
vitro. Exp

cally-evoked granule cell responses in hippocampal slices. Later studies extended these observations by showing that ACh applied in the pyramidal cell layer increased and prolonged EPSPs, whereas dendritic application had a suppressing effect on EPSPs and on population spikes.[14,30] Furthermore, in recent publications, ACh has been reported to exert a powerful disinhibitory effect *in situ*[18,22] as well as in hippocampal slices.[30] The present results are consistent with the hypothesis that part of the action of ACh in the hippocampus is mediated at a presynaptic site, since the potency of IPSPs was strongly reduced when ACh was either applied to the bath or iontophoretically-ejected onto the perisomatic region of the recorded neuron. Postsynaptic effects such as changes in membrane input resistance, block of chloride channels or of GABA receptors can, however, not be excluded. The amplitude of EPSPs was also decreased, but it was again not clear whether this reduction was due to membrane depolarization or consecutive to a presynaptic effect of ACh on excitatory nerve terminals. As shown also by other investigators,[18,30] the presumed presynaptic action of ACh had a more rapid time course than the depolarizing effect of ACh.

Paroxysomal depolarization shifts

During bath application and during distant iontophoretic administration of ACh at high current strength, pyramidal cells often produced paroxysmal depolarization shifts accompanied by bursting discharges (see Fig. 2B, 4D and 4E). Such epileptiform activity was not observed during bath administration of L-glutamate (Fig. 2A), a drug which also excited and depolarized pyramidal cells. Similar depolarization shifts, however, were previously observed in response to bath application of γ-aminobutyrate (GABA)-antagonists[6] or opiate receptor agonists.[6,8] The PDS induced by ACh apparently involve receptors different from GABA or opiate receptors, since naloxone, a specific opiate antagonist, did not influence the ACh response and since ACh (R. Maurer, personal communication) and cholinergic drugs[32] have a low affinity for GABA-binding sites.

The synthetic enkephalin analogue FK 33-824 had no influence on pyramidal cells in TTX-treated cultures (see also Haas & Gähwiler[10]), whereas ACh still induced PDS under these conditions. The latter finding not only supports the hypothesis that pyramidal cells possess intrinsic burst-generating mechanisms,[24a] but it also shows that these mechanisms can be triggered by a neurotransmitter acting postsynaptically.

Cholinergic inputs to the hippocampus are thought to originate mainly in the medial septal nuclei.[20,21,26,29] It is, therefore, noteworthy that the present study shows ACh receptors to be retained following long-term culture of an isolated *in vitro* preparation. Our data suggest that ACh receptors, once established, remain present and functional for prolonged periods even in the absence of their physiological ligand. In contrast, Rothman[23] has shown that ACh had no effect in cultures of dispersed fetal hippocampal neurons, where cholinergic connections presumably had not yet been established at the time of cultivation.

Acknowledgements—We thank Drs P. Herrling and E. van Deusen for valuable discussions and Miss E. Hoffmann for excellent technical assistance.

REFERENCES

1. Benardo L. S. & Prince D. A. (1981) Acetylcholine induced modulation of hippocampal pyramidal neurons. *Brain Res.* **211**, 227–234.
2. Bird S. J. & Aghajanian G. K. (1975) Denervation super-sensitivity in the cholinergic septo-hippocampal pathway: a micro-iontophoretic study. *Brain Res.* **100**, 355–370.
3. Biscoe T. J. & Straughan D. W. (1966) Micro-electrophoretic studies of neurones in the cat hippocampus. *J. Physiol., Lond.* **183**, 341–359.
4. Bland B. H., Kostopoulos G. K. & Phillis J. W. (1974) Acetylcholine sensitivity of hippocampal formation neurons. *Can. J. Physiol. Pharmac.* **52**, 966–971.
5. Dodd J., Dingledine R. & Kelly J. S. (1981) The excitatory action of acetylcholine on hippocampal neurones of the guinea-pig and rat maintained *in vitro*. *Brain Res.* **207**, 109–127.
6. Gähwiler B. H. (1980) Excitatory action of opioid peptides and opiates on cultured hippocampal pyramidal cells. *Brain Res.* **194**, 193–203.
7. Gähwiler B. H. (1981) Organotypic monolayer cultures of nervous tissue. *J. Neurosci. Meth.* **4**, 329–342.
8. Gähwiler B. H. & Herrling P. L. (1981) Effects of opioid peptides on synaptic potentials in explants of rat hippocampus. *Reg. Pept.* **1**, 317–326.
9. Haas H. L. (1982) Cholinergic disinhibition in hippocampal slices of the rat. *Brain Res.* **233**, 200–204.
10. Haas H. L. & Gähwiler B. H. (1980) Do enkephalins directly affect calcium-spikes in hippocampal pyramidal cells? *Neuroscience Letters* **19**, 89–92.
11. Herrling P. L. (1981) The membrane potential of cat hippocampal neurons recorded *in vivo* displays four different reaction mechanisms to iontophoretically-applied transmitter agonists. *Brain Res.* **212**, 331–343.
12. Herrling P. L. & Gähwiler B. H. (1981) High-frequency stimulation of hippocampal pyramidal cells *in situ* and *in vitro*. *Experientia* **37**, 610.

13. Herz A. & Nacimiento A. C. (1965) Ueber die Wirkung von Pharmaka auf Neurone des Hippocampus nach mikroelektrophoretischer Verabfolgung. *Naunyn-Schmiedebergs Arch. Pharmac.* **251**, 295–314.
14. Hounsgaard J. (1978) Presynaptic inhibitory action of acetylcholine in area CA1 of the hippocampus. *Expl. Neurol.* **62**, 787–797.
15. Kelly J. S., Dodd J. & Dingledine R. (1979) Acetylcholine as an excitatory and inhibitory transmitter in the mammalian central nervous system. *Prog. Brain Res.* **49**, 253–266.
16. Knowles W. D. & Schwartzkroin P. A. (1981) Local circuit synaptic interactions in hippocampal brain slices. *J. Neurosci.* **1**, 318–322.
17. Krnjević K., Pumain R. & Renaud L. (1971) The mechanisms of excitation by acetylcholine in the cerbral cortex. *J. Physiol., Lond.* **215**, 447–465.
18. Krnjević K., Reiffenstein R. J. & Ropert N. (1980) Disinhibitory action of acetylcholine in the hippocampus. *J. Physiol., Lond.* **308**, 73P–74P.
19. MacVicar B. A. & Dudek F. E. (1980) Local synaptic circuits in rat hippocampus: interactions between pyramidal cells. *Brain Res.* **184**, 220–223.
20. Mellgren S. I. & Srebro B. (1973) Changes in acetylcholinesterase and distribution of degenerating fibres in the hippocampal region after septal lesions in the rat. *Brain Res.* **52**, 19–36.
21. Mosco S., Lynch G. & Cotman C. (1973) The distribution of septal projections to the hippocampus of the rat. *J. comp. Neurol.* **152**, 163–174.
22. Reiffenstein R. J., Krnjević K. & Ropert N. (1980) Acetylcholine has a powerful disinhibitory action in the hippocampus. *Soc. Neurosci. Abs.* **6**, 514.
23. Rothman S. M. (1980) Development of transmitter sensitivity of dispersed fetal hippocampal neurons in culture. *Soc. Neurosci. Abs.* **6**, 290.
24. Schwartzkroin P. A. & Slawsky M. (1977) Probable calcium spikes in hippocampal neurons. *Brain Res.* **135**, 157–161.
24a. Schwartzkroin P. A. & Wyler A. R. (1979) Mechanisms underlying epileptiform burst discharge. *Annals of Neurol.* **7**, 95–107.
25. Segal M. (1978) The acetylcholine receptor in the rat hippocampus; nicotinic, muscarinic or both? *Neuropharmacology* **17**, 619–623.
26. Segal M. & Landis S. (1974) Afferents to the hippocampus of the rat studied with the method of retrograde transport of horseradish peroxidase. *Brain Res.* **78**, 1–15.
27. Stefanis C. (1964) Hippocampal neurons: their responsiveness to microelectrophoretically-administered endogenous amines. *Pharmacologist* **6**, 171.
28. Steiner T. A. (1968) Influence of microelectrophoretically-applied acetylcholine on responsiveness of hippocampal and lateral geniculate neurons. *Pfluegers Arch. ges. Physiol.* **303**, 173–180.
29. Straughan D. W. (1975) Neurotransmitters and the hippocampus. In *The Hippocampus* (eds Isaacson R. L. & Pribram K. H.) Vol. 1, pp. 239–268. Plenum Press, New York.
30. Valentino R. J. & Dingledine R. (1981) Presynaptic inhibitory effect of acetylcholine in the hippocampus. *J. Neurosci.* **1**, 784–792.
31. Yamamoto C. & Kawai N. (1967) Presynaptic action of acetylcholine in thin sections from the guinea-pig dentate gyrus *in vitro*. *Expl Neurol.* **19**, 176–187.
32. Zukin S. R., Young A. B. & Snyder S. H. (1974) Gamma-aminobutyric acid binding to receptor sites in the rat central nervous system. *Proc. natn. Acad. Sci. U.S.A.* **71**, 4802–4807.

(*Accepted* 13 *December* 1981)

Neuroscience Vol. 7, No.
Printed in Great Britain

S
O
H

Abstract-
neuronal
mitter m
by a redu
and chol
uptake c
caudal k
in the pi
effect on
The re
dependei
The neu

Kainic acid, giv
cally, induces m
areas of rats.[6,7]
acid, a structur
probably relatec
in some sites bc
on a glutamerg
indicated that tl
of kainic acid
septum[22] and h
creased if the
ways are previc

The details
neurotoxicity c
stood, but evid
effects probabl
genic activity
seizure activity
known to dam
threshold.[3,4,23]
fact that diazep
elevate the th
against some o

In a previou
some neurotra
regions after sy
structures studi
daloid comple

Abbreviations: A
transferate; GA
dicarboxylase; H